



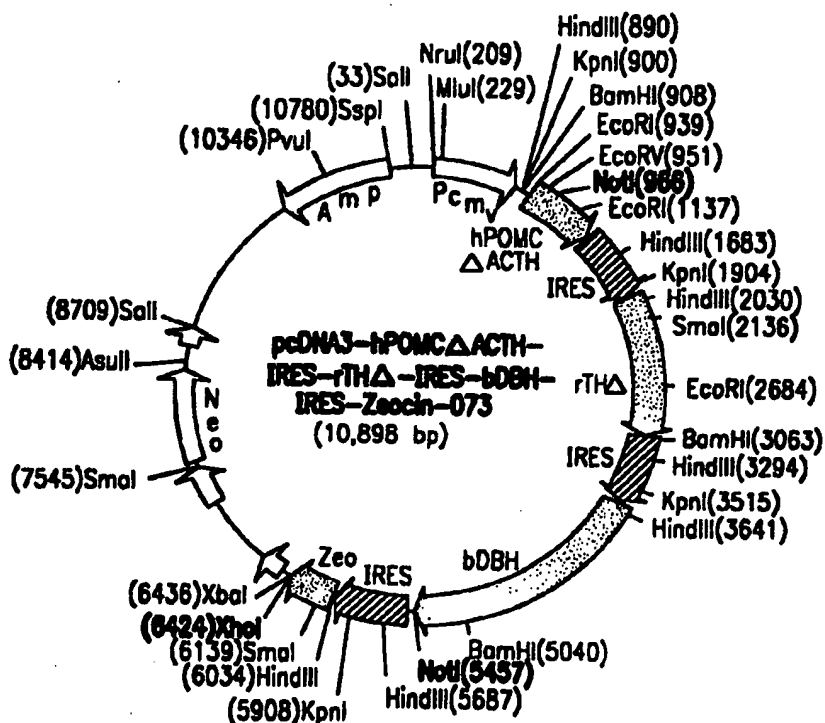
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(54) Title: CELL LINE PRODUCING ANALGESIC COMPOUNDS FOR TREATING PAIN

(57) Abstract

A genetically engineered cell line that produces at least one catecholamine, at least one endorphin, and at least one enkephalin, for the treatment of pain. The cells may be provided directly to a patient in need thereof, or encapsulated to form a bioartificial organ.



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Cell line producing analgesic compounds for treating pain

Field of the Invention

The present invention relates to a cell line
5 useful for the treatment of pain. More particularly,
the cell line of this invention has been genetically
engineered to produce at least one analgesic compound
from each of the groups consisting of endorphins,
enkephalins, and catecholamines.

10 Background of the Invention

Pain is a common symptom of disease. The
superficial dorsal horn of the spinal cord, where
primary afferent fibers carrying nociceptive
information terminate, contains enkephalinergic
15 interneurons and high densities of opiate receptors.
In addition, there is a dense concentration of
noradrenergic fibers in the superficial laminae of the
spinal cord.

Acute pain arises in response to acute
20 noxious stimuli. Chronic pain is predominantly due to
neuropathies of central or peripheral origin. This

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neuropathic pain is the result of aberrant somatosensory processing that can result in increased sensitivity to a painful stimulus (hyperalgesia) and pain associated with a stimulus that does not usually
5 provoke pain (allodynia).

Intrathecal injection of morphine into the spinal subarachnoid space produces potent analgesia. Similarly, intrathecal administration of norepinephrine or noradrenergic agonists also produces analgesia.

10 See, e.g., Sagen et al., Proc. Natl. Acad. Sci. USA, 83, pp. 7522-26 (1986).

Co-administration of subeffective doses of opiates, such as enkephalins, and catecholamines, such as norepinephrine, may synergize to produce analgesia.
15 Ibid. Chromaffin cells in the adrenal medulla produce and release several neuroactive substances including norepinephrine, epinephrine, met-enkephalin, leu-enkephalin, neuropeptide Y, vasoactive intestinal polypeptide, somatostatin, neurotensin, cholecystokinin
20 and calcitonin gene-related peptide. See, e.g., Sagen et al., Proc. Natl. Acad. Sci. USA, 83, pp. 7522-26 (1986); Sagen et al., Jour. Neurochem., 56, pp. 623-27 (1991).

Because chromaffin cells produce both opioid
25 peptides and catecholamines, one approach to reduction of nociceptive response or pain sensitivity has investigated transplanting adrenal medullary tissue, as well as isolated adrenal chromaffin cells, directly into CNS pain modulatory regions, in attempts to
30 provide analgesia. See, e.g., Sagen et al., Brain Research, 384, pp. 189-94 (1986); Vaguero et al., Neuroreport, 2, pp. 149-51 (1991); Ginzberg and

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Seltzer, Brain Research, 523, pp. 147-50 (1990); Sagen et al., Pain, 42, pp. 69-79 (1990).

Attempts to produce analgesic have been made using both allogeneic and xenogeneic chromaffin tissue or cells transplants. Allograft tissue is in limited supply, and is not readily available, particularly for in human pain treatment programs. In addition, allogeneic human tissue carries the risk of pathogenic contamination. See e.g., Hama and Sagen, Brain Research, 651, pp. 183-93 (1994).

Xenogeneic donors may provide large quantities of material that can be readily obtained. For this reason, bovine adrenal tissue has been used. See, e.g., Hama and Sagen, Brain Research, 651, pp. 183-93 (1994).

However, potentially serious host consequences, as well as ultimate graft rejection, are inherent problems in transplantation between disparate species. Complete graft rejection of whole or dissociated tissue may occur even in the CNS, normally thought to be immunologically privileged, due to presence of highly antigenic cells in the xenografts, particularly endothelial cells. In addition, the donor tissue must be carefully screened to avoid introduction of viral contaminants, or other pathogens, to the host. To overcome graft rejection, immunosuppression is required typically using cyclosporine A.

Some reduction in pain sensitivity has been reported resulting from these transplants, particularly for the reduction of low intensity chronic pain. In most reports, significant differences between control and transplanted animals were noted only after nicotine

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administration to stimulate opioid peptide production. However, there have been some reports that analgesia has been observed in a rat chronic pain model from basal level activity of chromaffin tissue allografts.

- 5 See, e.g., Vaquero et al., NeuroReport, 2, pp. 149-51 (1991) and Hama and Sagen, Brain Research, 651, pp. 183-93 (1994).

Bovine adrenal chromaffin cells have been encapsulated to form a bioartificial organ ("BAO") for
10 implantation into rats for the treatment of acute and chronic pain. See, e.g., Sagen et al., J. Neurosci., 13, pp. 2415-23 (1993) and Hama et al., 7th World Congress Pain, Abstract 982, Paris France (1993). Initial trials in human subject have been conducted
15 using encapsulated bovine chromaffin cells. See, Aebischer et al., Transplantation, 58, pp. 1275-77 (1994).

There have also been attempts to induce antinociception using other cells, e.g., AtT-20 cells.
20 AtT-20 cells were originally derived from a mouse anterior pituitary tumor. These cells synthesize and secrete β -endorphin. See, e.g., Wu et al., J. Neural Transpl. & Plasticity, 5, pp. 15-26 (1993).

AtT-20/hENK cells are AtT-20 cells that have been
25 genetically engineered to carry the entire human pro-enkephalin A gene (i.e. containing 6 met-enkephalin sequences and one leu-enkephalin sequence) with 200 bases of 5'-flanking sequence and 2.66 kilobases of 3'-flanking sequence. See Wu et al., supra, Comb et al.,
30 EMBO J., 4, pp. 3115-22 (1985).

Wu et al., J. Neural Transpl. & Plasticity, 5, pp. 15-26 (1993) refers to rat hosts transplanted

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with AtT-20 or AtT-20/hENK cells. Unstimulated AtT-20/hENK cells produced more antinociception (tail flick test) than produced by AtT-20 implants. In contrast, isoproterenol stimulation produced more antinociception with AtT-20 cells than with AtT-20/hENK cells. Ibid.

In mice hosts, AtT-20 or AtT-20/hENK implants did not affect basal response to thermal nociceptive stimuli. Mice receiving AtT-20 implants developed tolerance to β -endorphin and a μ -opioid agonist (DAMGO). Mice receiving AtT-20/hENK implants developed tolerance to an δ -opioid agonist (DPDPE). In response to repeated doses of an μ opiate agonist, mice receiving AtT-20/hENK implants developed less tolerance compared to mice receiving AtT-20 cells or controls.

The antinociceptive effect of isoproterenol treatment appeared equal in mice receiving AtT-20 or AtT-20/hENK cell implants. See, Wu et al., J. Neuroscience, 14, pp. 4806-14 (1994). Wu et al. speculated that one reason for the absence of additional antinociception in mice implanted with enkephalin producing AtT-20/hENK cells may be due to lack of sensitivity of the behavioral assays. Another possible reason was that met-enkephalin's known antagonist effect on morphine induced antinociception offset the potentiating effect of the single leu-enkephalin, particularly since there are 6 met-enkephalin sequences for each leu-enkephalin sequence in pro-enkephalin A.

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Summary of the Invention

The present invention provides a cell line that has been genetically engineered to produce at least one analgesic compound from each of the groups
5 consisting of endorphins, enkephalins, and catecholamines. The cell line may be used in the treatment of pain.

There are advantages to using a cell line over the use of primary cells. Expensive and time
10 consuming testing to ensure safety and performance criteria for cells must be performed for individual isolations of primary cells. Less testing is required of a cell bank. There is no need to isolate primary cells. Output of the desired analgesics may be more
15 stable since the performance of primary cells may be dependent on the age, sex, health or hormonal status of the donor animal. It is also possible to achieve higher output of the desired products, as well as to engineer specifically modified peptides into the cell
20 line. This permits delivery of multiple analgesics simultaneously. Expression of one or more of the analgesics can be regulated (by using a regulatable promoter to drive expression). In addition, for safety, a "suicide" gene can be incorporated into the
25 cell line. Further, for encapsulation purposes proliferating cells have the advantage that they divide to replace dying or dead cells.

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Brief Description of the Drawing

Figure 1 is a plasmid map of vector pBS-hPOMC-027, pBS-IgSP-hPOMC-028 and pBS-IgSP-hPOMC- Δ ACTH-029.

5 Figure 2 is a plasmid map of vectors pCEP4-hPOMC-030, pCEP4-hPOMC-031, pcDNA3-hPOMC-034 and pcDNA3-hPOMC-035.

 Figure 3 is a plasmid map of vectors pCEP4-hPOMC- Δ ACTH-032, pCEP4-hPOMC- Δ ACTH-033, pcDNA3-hPOMC-
10 Δ ACTH-36 and pcDNA3-hPOMC- Δ ACTH-037.

 Figure 4 is a plasmid map of vectors pcDNA3-rTH-044, pcDNA3-rTH Δ -045, and pcDNA3-rTHDKS-075 (also represented as pcDNA3-rTH Δ KS-075).

 Figure 5 is a plasmid map of vectors pcDNA3-
15 rTH Δ -IRES-bDBH-088 and pcDNA3-rTH Δ KS-IRES-bDBH-076.

 Figure 6 is a plasmid map of vector pZeo-Pcmv-rTH Δ KS-IRES-bDBH-088.

 Figure 7 is a plasmid map of vector pBS-Pcmv-rTH Δ IRES-bDBH-067.

20 Figure 8 is a plasmid map of vector pBS-hPOMC- Δ ACTH-IRES-rTH Δ IRES-bDBH-068.

 Figure 9 is a plasmid map of vector pcDNA3-hPOMC- Δ ACTH-IRES-rTH Δ -IRES-bDBH-069.

 Figure 10 is a plasmid map of vector pcDNA3-
25 IRES-Zeocin-072.

 Figure 11 is a plasmid map of vector pcDNA3-hPOMC- Δ ACTH-IRES-rTH Δ -IRES-bDBH-IRES-Zeocin-073.

 Figure 12 is a plasmid map of vector pcDNA3-hPROA+KS-091.

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Detailed Description of the Invention

In order that this invention may be more fully understood, the following detailed description is set forth.

5 Any suitable cell may be transformed with the recombinant DNA molecules of this invention. Among the contemplated cells are chromaffin cells, including conditionally immortalized chromaffin cells such as those described in WO 96/02646, Neuro-2A, PC12, PC12a,
10 SK-N-MC, AtT-20, and RIN cells including RINa and RINb. Preferably the cell has endogenous prohormone convertases and/or dopa decarboxylases.

SK-N-MC cells, a neuroepithelioma cell line, co-expresses several neuropeptides, including
15 enkephalin, cholecystokinin and gastrin-releasing peptide. See, e.g., Verbeeck et al., J. Biol. Chem., 265, pp. 18087-090 (1990). The pro-enkephalin A gene has been expressed in SK-N-MC cells. See, e.g., Folkesson et al., Mol. Brain Res., 3, pp. 147-54
20 (1988). We prefer AtT-20 and RIN cells, most preferably RIN cells.

RIN cells are a pancreatic endocrine cell line derived from rat. See, e.g., Horellou et al., J. Physiol., 85, pp. 158-70 (1991). RIN cells are
25 known to endogenously produce GABA and β -endorphin.

Some of the characteristics of various contemplated cells are shown in Table 1.

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Table 1

	<u>Cells</u>	<u>Analgesic Substances</u>	<u>Other Components</u>
	Chromaffin	NE, met-enkephalin	TH, DDC, D β H, PC
	PC12, PC12a	low NE & met-enkephalin	DDC, D β H, PC
5	AtT-20	β -endorphin	DDC, PC
	RINa	β -endorphin, GABA	DDC, PC
	RINb	β -endorphin	DDC, PC
	Neuro 2A		DDC, D β H, PC
	TH =	Tyrosine hydroxylase converts tyrosine - l-dopa	
10	DDC =	Dopamine decarboxylase converts l-dopa - dopamine (DA)	
	D β H =	Dopamine β -Hydroxylase converts DA - norepinephrine (NE)	
	PC =	Prohormone Convertases process POMC to β -endorphin and Pro-enkephalin A (ProA) to met-enkephalin.	
	AtT20 =	Mouse pituitary corticotroph cell line that endogenously secretes β -endorphin	
15	RIN =	Rat insulinoma	
	Neuro 2A =	Mouse neuroblastoma	

The primary delivery products include at least one each of an endorphin, an enkephalin and a catecholamine.

Enkephalins and endorphins are endogenous opioid peptides in humans. These opioid peptides comprise approximately 15 compounds ranging from 5 to 31 amino acids. These compounds bind to and act at least in part via the same μ opioid receptor as morphine, but are chemically unrelated to morphine. In addition, these compounds stimulate other opiate receptors. Yaksh and Malmberg, Textbook of Pain, 3rd Ed. (Eds. P. Wall and R. Melzack), "Central Pharmacology of Nociceptive Transmission," pp. 165-200, 1994 (New York).

The opioid peptides have common chemical properties, but are synthesized in different pathways.

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β -endorphin, the most abundant endorphin, is synthesized as part of a larger precursor molecule, pro-opiomelanocortin ("POMC"). The POMC molecule contains the full sequence of adrenocorticotrophic hormone ("ACTH"), α -melanocyte-stimulating hormone (" α -MSH"), β -MSH, and β -lipotropin. The POMC precursor molecule also has the potential to generate other endorphins, including α -endorphin and gamma-endorphin. Processing of the POMC precursor occurs differently within various tissues according to the localization of cleavage enzymes, such as prohormone convertases, within those tissues.

In the pituitary, POMC is cleaved to produce ACTH and β -endorphin, and the ACTH is not further processed. In contrast, in the hypothalamus, ACTH is converted to β -MSH. While different cell types may synthesize the same primary gene product, the final profile of hormone secretion may differ widely.

This invention contemplates use of a DNA sequence encoding any suitable endorphin that has analgesic activity. In addition, analogs or fragments of these endorphins that have analgesic activity are also contemplated. Thus the endorphin to be produced by the cells of this invention may be characterized by amino acid insertions, deletions, substitutions and modifications at one or more sites in the naturally occurring amino acid sequence of the desired endorphin. We prefer conservative modifications and substitutions (i.e., those having a minimal effect on the secondary or tertiary structure of the endorphin and on the analgesic properties of the endorphin). Such conservative substitutions include those described by

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Dayhoff in Atlas of Protein Sequence and Structure, 5, (1978) and by Argos, Embo J., 3, pp. 779-85 (1989).

Techniques for generating such variants of naturally occurring endorphins are well known. For example, codons in the DNA sequence encoding the wild type endorphin may be altered by site specific mutagenesis.

This invention contemplates using a DNA sequence encoding the entire POMC precursor molecule. This embodiment takes advantage of the host cell's cleavage enzymes (i.e., Prohormone convertase 2) to generate a suite of endorphins, some or all of which may have analgesic properties.

This invention also contemplates use of DNA
15 fragments of the POMC gene that encode a particular
desired endorphin.

The DNA and amino acid sequence of POMC are well known. Cochet et al., Nature, 297, pp. 335-9 (1982); Takahashi et al., Nucl. Acids Res., 11, pp. 6847-58 (1983).

We prefer a DNA sequence encoding POMC in which the ACTH coding region has been deleted. The preferred endorphin encoded by this construct is β -endorphin.

25 Some enkephalins are synthesized in the
adrenal glands as part of a large protein, pro-
enkephalin A, that contains six repeats of the Met-
enkephalin sequence and one Leu-enkephalin structure.
Met-enkephalin, as well as Met-enkephalin-Arg-Phe and
30 Met-enkephalin-Arg-Gly-Leu have significant
antinociceptive activity. See, e.g., Sagen et al.,
Brain Res., 502, pp. 1-10 (1989).

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Other enkephalins, i.e., dynorphins and neo-endorphins are derived from a distinct molecule, pro-enkephalin B. Additional "cryptic" peptides are also encoded within the structure of these precursor
5 proteins, and may be released by "pro-hormone-type" cleavage. See, e.g., Harrison's "Principles Of Internal Medicine", 12th Edition, pp. 1168-69 (1991).

This invention contemplates use of a DNA sequence encoding any suitable enkephalin that has
10 analgesic activity. Analogs and active fragments that have analgesic properties are also contemplated. Such analogs or fragments may thus have amino acid insertions, deletions, substitutions at one or more sites in the naturally occurring amino acid sequence.
15 Such variants may be generated as described above.

This invention contemplates use of a DNA sequence encoding a desired enkephalin in its "mature" form. In addition, this invention contemplates using a DNA sequence encoding the entire pro-enkephalin A
20 precursor, or the entire pro-enkephalin B precursor. Further, we also contemplate using DNA encoding a fusion, or fragment of these sequences, that upon expression yields one or more enkephalin-like molecules that have analgesic properties.

25 We prefer use of a DNA sequence encoding the entire pro-enkephalin A precursor molecule. The DNA and amino acid sequence of pro-enkephalin A are well known. Folkesson, supra. This embodiment takes advantage of the host cell's cleavage enzymes, such as
30 prohormone convertase, to generate a suite of enkephalins, some or all of which may have analgesic

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properties. The preferred enkephalin encoded by this construct is Met-enkephalin.

There are three naturally occurring catecholamines which function as neurotransmitters in the central nervous system; norepinephrine ("NE"), epinephrine ("E"), and dopamine. NE is associated with postganglionic sympathetic nerve endings. NE exerts its effects locally in the immediate vicinity of its release.

10 Catecholamines are synthesized from the amino acid tyrosine, which is sequentially hydroxylated to form dihydroxyphenylalanine (dopa), decarboxylated to form dopamine, and then hydroxylated on the beta position of the side chain by dopamine beta hydroxylase
15 to form NE. Harrison's, supra, pp. 380. NE is N-methylated to E by phenylethanolamine-N methyltransferase ("PNMT").

Hydroxylation of tyrosine by tyrosine hydroxylase ("TH") is the rate limiting step in NE
20 synthesis. Regulation of dopa and NE synthesis in the adrenal medulla may be accomplished by changes in the amount and the activity of TH.

In addition, regulation of synthesis of E from NE may occur by changes in the amount and the
25 activity of phenylethanolamine-N-methyltransferase ("PNMT"). PNMT is inducible by glucocorticoids from the adrenal cortex. Ibid.

Catecholamines are maintained in high concentration in adrenal medullary chromaffin tissue,
30 mostly as E. Opioid peptides are also stored in the adrenal gland.

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NE and E have similar affinities at α_2 receptors and therefore both potentially contribute to analgesia. Bylund, FASEB J., 6, PP. 832-39 (1992). The enkephalin peptides that predominantly include met-

5 enkephalin selectively activate delta (δ) opioid receptors. Reisine and Bell, Trends Neurosci., 16, pp. 506-10 (1993). Activation of α_2 adrenergic and δ opioid receptors in the spinal cord each result in antinociception and are potentially synergistic. Yaksh

10 and Malmberg, Progress in Pain Research and Management, Vol. 1, Ed. Fields and Lisbeskind, IASP Press, Seattle, pp. 141-71 (1994). Activation of δ versus (μ) opioid receptors in experimental animals results in fewer adverse side effects including constipation and

15 addiction liability (Lee et al., J. Pharmacol. Exp. Ther., 267, pp. 883-87 (1993). The combined delivery of different opioidergic and adrenergic agents may decrease the magnitude of tolerance that develops to a single agent and lead to sustained pain relief. Yaksh

20 and Reddy, Anesthesiol., 54, pp. 451-67 (1981).

This invention contemplates use of a DNA sequence encoding catecholamine biosynthetic enzymes or analogs or fragments thereof to obtain catecholamines that have analgesic properties. The preferred

25 catecholamines in this invention are NE and E.

In one embodiment, the host cell is transformed with the genes necessary to accomplish production of NE or E, as desired. The selection of heterologous gene sequences required depends upon the

30 complement of catecholamine synthesizing enzymes normally occurring in the host cell. For example, RIN cells, and AtT-20 cells lack tyrosine hydroxylase

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("TH") and dopamine beta hydroxylase ("DBH"). However, RIN and AtT-20 cells contain endogenous dopa decarboxylase ("DDC"). If the desired catecholamine is E, then the gene encoding PNMT is also required. The
5 gene encoding PNMT is known. Baetge et al., Proc. Nat'l Acad. Sci., 83, pp. 5455-58 (1986).

The gene encoding TH is known. See, e.g., United States patent 5,300,436, incorporated herein by reference. Modified TH variants are also known.
10 United States patent 5,300,436. In addition, truncated versions of TH that contain the necessary C-terminal catalytic domains are also known. See, e.g., Daubner et al., Protein Science, 2, pp. 1452-60 (1993).

AtT-20 cells have been transformed with wild
15 type TH, as well as various TH muteins. See, e.g., Wu et al., J. Biol. Chem., 267, pp. 25754-758 (1992).

The sequence of the DBH gene is also well known. See, e.g., Lamoroux et al., EMBO J., 6, pp. 3931-37 (1987).

20 It will be appreciated that in addition to the preferred DNA sequences described herein, there will be many degenerate DNA sequences that code for the desired analgesics.

Secondary compounds with potential analgesic
25 action may also be produced by the cells of this invention. Such compounds include galanin and somatostatin. In addition, neuropeptide Y, neurotensin and cholecystokinin may be produced by the transformed cells of this invention. The cells of this invention
30 may normally produce some or all of these compounds, or may be genetically engineered to do so using standard techniques.

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Standard methods may be used to obtain or synthesize the genes encoding the analgesic compounds to be produced by the cells of this invention.

For example, the complete amino acid sequence
5 of the desired compound may be used to construct a back-translated gene. A DNA oligomer containing a nucleotide sequence coding for the desired analgesic compound may be synthesized. For example, several small oligonucleotides coding for portions of each
10 desired polypeptide may be synthesized and then ligated. The individual oligonucleotides typically contain 5' or 3' overhangs for assembly.

The DNA sequence encoding each desired analgesic compound, may or may not also include DNA
15 sequences that encode a signal sequence. Such signal sequence, if present, should be one recognized by the cell chosen for expression of the analgesic compound. It may be prokaryotic, eukaryotic or a combination of the two. It may also be the signal sequence of the
20 native compound. It generally is preferred that a signal sequence be encoded and most preferably that the native signal sequence be used.

Once assembled, the DNA sequences encoding the desired compounds will be inserted into one or more
25 expression vectors and operatively linked to expression control sequences appropriate for expression in the desired transformed cell.

Proper assembly may be confirmed by nucleotide sequencing, restriction mapping, and
30 expression of a biologically active polypeptide in the transformed cell. As is well known in the art, in order to obtain high expression levels of a transfected

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gene in a host, the gene must be operatively linked to transcriptional and translational expression control sequences that are functional in the chosen expression cell.

5 The choice of expression control sequence and expression vector will depend upon the choice of cell. A wide variety of expression host/vector combinations may be employed. Useful expression vectors for eukaryotic hosts, include, for example, vectors
10 comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus.

We prefer pcDNA3, pCEP4, pZeoSV (InVitrogen, San Diego) and pNUT.

Any of a wide variety of expression control
15 sequences may be used in these vectors. Such useful expression control sequences include the expression control sequences associated with structural genes of the foregoing expression vectors. Examples of useful expression control sequences include, for example, the
20 early and late promoters of SV40 or adenovirus, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α -mating system and other sequences known to control the expression of
25 genes of eukaryotic cells or their viruses, and various combinations thereof.

It should of course be understood that not all vectors and expression control sequences will function equally well to express the DNA sequences
30 described herein. Neither will all cells function equally well with the same expression system. However, one of skill in the art may make a selection among

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these vectors, expression control sequences and cells without undue experimentation. For example, in selecting a vector, the host cell must be considered because the vector must replicate in it. The vector's
5 copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered.

In selecting an expression control sequence,
10 a variety of factors should also be considered. These include, for example, the relative strength of the sequence, its controllability, and its compatibility with the actual DNA sequence encoding the desired analgesic compounds, particularly as regards potential
15 secondary structures. Host cells should be selected by consideration of their compatibility with the chosen vector, the toxicity of the product coded for by the DNA sequences, their secretion characteristics, their ability to fold the polypeptides correctly, and their
20 culture requirements. If the host cell is to be encapsulated, cell viability when encapsulated and implanted in a recipient should also be considered.

Within these parameters, one of skill in the art may select various vector/expression control
25 sequence/host combinations that will express the desired DNA sequences in culture.

In one embodiment, cells (e.g., RIN cells) are sequentially transformed with 4 separate expression vectors containing the POMC gene, the pro-enkephalin A
30 gene, the TH gene and the DBH gene. In such a transformed host cell, amplification of copy number of the heterologous genes is more difficult to achieve.

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Thus use of fewer expression vectors is preferred. Most preferably, a single expression vector, containing all 4 heterologous genes, is used.

In a particular embodiment RIN cells are sequentially transformed with 3 expression vectors. The first vector contains the POMC gene operably linked to the CMV promoter. Preferably a truncated version of the POMC gene is used, having the ACTH coding region deleted. The second vector contains the pro-enkephalin A gene operably linked to the CMV promoter. Preferably the proA construct contains the Kozak sequence immediately upstream of the start codon. The third vector contains both the TH gene (preferably truncated and having the Kozak consensus sequence immediately upstream of the start codon) and the DBH gene. In this embodiment, the TH gene is operably linked to the CMV promoter. The DBH gene is operably linked to an internal ribosome entry site promoter sequence. RIN cells are then transformed sequentially with each expression vector according to known protocols.

In another embodiment, a single expression vector containing the pro-enkephalin A gene, the POMC gene, the TH gene, and the DBH gene is constructed. Preferably, the ACTH region of the POMC gene is deleted. Preferably the TH gene is truncated.

Multiple gene expression from a single transcript is preferred over expression from multiple transcription units. One approach for achieving expression of multiple genes from a single eukaryotic transcript takes advantage of sequences in picorna viral mRNAs known as internal ribosome entry sites ("IRES"). These sites function to facilitate protein

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translation from sequences located downstream from the first AUG of the mRNA.

Macejak and Sarnow reported that the 5' untranslated sequence of the immunoglobulin heavy chain binding protein (BiP, also known as CRP 78, the glucose-regulated protein of molecular weight 78,000) mRNA can directly confer internal ribosome binding to an mRNA in mammalian cells, in a 5'-cap independent manner, indicating that translation initiation by an internal ribosome binding mechanism is used by this cellular mRNA. Nature 353, pp. 90-94 (1991).

WO 94/24870 refers to use of more than two IRES for translation initiation from a single transcript, as well as to use of multiple copies of the same IRES in a single construct.

This invention also contemplates use of a "suicide" gene in the transformed cells. Most preferably, the cell carries the TK (thymidine kinase) gene as a safety measure, permitting the host cell to be killed in vivo by treatment with gancyclovir.

Use of a "suicide" gene is known in the art. See, e.g., Anderson, published PCT application WO 93/10218; Hamre, published PCT application WO 93/02556. The recipient's own immune system provides a first level of protection from adverse reactions to the implanted cells. If encapsulated, the polymer capsule itself may be immuno-isolatory. The presence of the TK gene (or other suicide gene) in the expression construct adds an additional level of safety to the recipient of the implanted cells.

Preferred vectors for use in this invention include those that allow the DNA encoding the analgesic

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compounds to be amplified in copy number. Such amplifiable vectors are well known in the art. They include, for example, vectors able to be amplified by DHFR amplification (see, e.g., Kaufman, United States Patent 4,470,461, Kaufman and Sharp, "Construction Of A Modular Dihydrofolate Reductase cDNA Gene: Analysis Of Signals Utilized For Efficient Expression", Mol. Cell. Biol., 2, pp. 1304-19 (1982)) or glutamine synthetase ("GS") amplification (see, e.g., United States patent 5,122,464 and European published application 338,841). Such amplification can be used to increase output of the desired analgesic compounds.

Other techniques for increasing the output of the desired analgesic compounds are contemplated. For example, subcloning existing polyclonal cell lines is contemplated. Cells are cloned by limiting dilution to a single cell in each well. Cell clones are cultures, and the clones are tested to select the clone with the highest output of analgesic substances.

Another technique for increasing the output of the desired analgesic compounds involves cloning altered forms of biosynthetic enzymes with higher activity than the wild type form (i.e., the truncated TH 1-155). Some truncated forms of TH have 4-6 times increased activity over the wild type form of TH. See, e.g., Daubner et al., "Expression and characterization of catalytic and regulatory domains of rat tyrosine hydroxylase" Protein Science, 2, pp. 1452-60 (1993).

In addition, use of tyrosine-free media to select to increase tetrahydrobiopterin cofactor levels may potentially increase tyrosine hydroxylase activity. See, e.g., Horellou et al., "Retroviral transfer of a

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human tyrosine hydroxylase cDNA in various cell lines; regulated release of dopamine in mouse anterior pituitary AtT-20 cells", Proc. Natl. Acad. Sci. USA, 86, pp. 7233-37 (1989).

5 Preferably, the output of β -endorphin ranges between 1 and 10,000 pg/ 10^6 cells/hr. Preferably, the output of met-enkephalin ranges between 1 and 10,000 pg/ 10^6 cells/hr. Preferably, the output of catecholamines ranges between 1 and 1,000 pmoles/ 10^6
10 cells/hr.

 The cells of this invention may be implanted into a mammal, including a human, for the treatment of pain. If implanted unencapsulated, any suitable implantation protocol may be used, including those
15 outlined by Sagen et al., United States patent 4,753,635, incorporated herein by reference.

 It may be desirable to encapsulate the genetically modified cells of this invention before implantation. Such encapsulated cells form a
20 bioartificial organ ("BAO"). BAOs may be designed for implantation in a recipient or can be made to function extra-corporeally. The BAOs useful in this invention typically have at least one semipermeable outer surface membrane or jacket surrounding a cell-containing core.
25 The jacket permits the diffusion of nutrients, biologically active molecules and other selected products through the BAO. The BAO is biocompatible.

 In some cases, the membrane may serve to also immunoisolate the cells by blocking the cellular and
30 molecular effectors of immunological rejection. The use of immunoisulatory membranes allows for the implantation of allo and xenogeneic cells into an

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individual without the use of immunosuppression. If biologically active molecules are released from the isolated cells, they pass through the surrounding semipermeable membrane into the recipient's body. If
5 metabolic functions are provided by the isolated cells, the substances to be metabolized enter the BAO from the recipient's body through the membrane to be acted on by the cells.

A variety of types of membranes have been
10 used in the construction of BAOs. Generally, the membranes used in BAOs are either microporous or ultrafiltration grade membranes. A variety of membrane materials have been suggested for use in BAOs, including PAN/PVC, polyurethanes, polysulfones,
15 polyvinylidienes, and polystyrenes. Typical membrane geometries include flat sheets, which may be fabricated into "sandwich" type constructions, having a layer of living cells positioned between two essentially planar membranes with seals formed around the perimeter of the
20 device. Alternatively, hollow fiber devices may be used, where the living cells are located in the interior of a tubular membrane. Hollow fiber BAOs may be formed step-wise by loading living cells in the lumen of the hollow fiber and providing seals on the
25 ends of the fiber. Hollow fiber BAOs may also be formed by a coextrusion process, where living cells are coextruded with a polymeric solution which forms a membrane around the cells.

BAOs have been described, for example, in
30 United States patent Nos. 4,892,538, 5,106,627, 5,156,844, 5,158,881, and 5,182,111, and PCT Application Nos. PCT/US/94/07015, WO 92/19195, WO

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93/03901, and WO 91/00119, all of which are incorporated herein by reference.

BAOs may contain other components that promote long term survival of the encapsulated cells.

5 For example, WO 92/19195 refers to implantable immunoisulatory biocompatible vehicles having a hydrogel matrix for enhancing cell viability.

The encapsulating membrane of the BAO may be made of a material which is the same as that of the
10 core, or it may be made of a different material. In either case, a surrounding or peripheral membrane region of the BAO which is permselective and biocompatible will be formed. The membrane may also be constructed to be immunoisulatory, if desired. The
15 core contains isolated cells, either suspended in a liquid medium or immobilized within a hydrogel matrix.

The choice of materials used to construct the BAO is determined by a number of factors and is described in detail in Dionne WO 92/19195. Briefly,
20 various polymers and polymer blends can be used to manufacture the capsule jacket. Polymeric membranes forming the BAO and the growth surfaces therein may include polyacrylates (including acrylic copolymers), polyvinylidenes, polyvinyl chloride copolymers,
25 polyurethanes, polystyrenes, polyamides, cellulose acetates, cellulose nitrates, polysulfones, polyphosphazenes, polyacrylonitriles, poly(acrylonitrile/covinyl chloride), as well as derivatives, copolymers and mixtures thereof.

30 BAOs may be formed by any suitable method known in the art. One such method involves coextrusion of a polymeric casting solution and a coagulant which

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can include biological tissue fragments, organelles, or suspensions of cells and/or other therapeutic agents, as described in Dionne, WO 92/19195 and United States Patents 5,158,881, 5,283,187 and 5,284,761,

5 incorporated herein by reference.

The jacket may have a single skin or a double skin. A single-skinned hollow fiber may be produced by quenching only one of the surfaces of the polymer solution as it is co-extruded. A double-skinned hollow
10 fiber may be produced by quenching both surfaces of the polymer solution as it is co-extruded.

Numerous capsule configurations, such as cylindrical, disk-shaped or spherical are possible.

The jacket of the BAO will have a pore size
15 that determines the nominal molecular weight cut off (nMWCO) of the permselective membrane. Molecules larger than the nMWCO are physically impeded from traversing the membrane. Nominal molecular weight cut off is defined as 90% rejection under convective
20 conditions. In situations where it is desirable that the BAO is immunoisulatory, the membrane pore size is chosen to permit the particular factors being produced by the cells to diffuse out of the vehicle, but to exclude the entry of host immune response factors into
25 the BAO. Typically the nMWCO ranges between 50 and 200 kD, preferably between 90 and 150 kD. The most suitable membrane composition will also minimize reactivity between host immune effector molecules known to be present at the selected implantation site, and
30 the BAO's outer membrane components.

The core of the BAO is constructed to provide a suitable local environment for the particular cells

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isolated therein. The core can comprise a liquid medium sufficient to maintain cell growth. Liquid cores are particularly suitable for maintaining transformed cell lines like PC12 cells. Alternatively, the core can comprise a gel matrix. The gel matrix may be composed of hydrogel (alginate, "Vitrogen™", etc.) or extracellular matrix components. See, e.g., Dionne WO 92/19195.

Compositions that form hydrogels fall into three general classes. The first class carries a net negative charge (e.g., alginate). The second class carries a net positive charge (e.g., collagen and laminin). Examples of commercially available extracellular matrix components include Matrigel™ and Vitrogen™. The third class is net neutral in charge (e.g., highly crosslinked polyethylene oxide, or polyvinylalcohol).

Any suitable method of sealing the BAO may be used, including the employment of polymer adhesives and/or crimping, knotting and heat sealing. These sealing techniques are known in the art. In addition, any suitable "dry" sealing method can also be used. In such methods, a substantially non-porous fitting is provided through which the cell-containing solution is introduced. Subsequent to filling, the BAO is sealed. Such a method is described in copending United States application Serial No. 08/082,407, herein incorporated by reference.

One or more in vitro assays are preferably used to establish functionality of the BAO prior to implantation in vivo. Assays or diagnostic tests well known in the art can be used for these purposes. See,

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e.g., Methods In Enzymology, Abelson [Ed], Academic Press, 1993. For example, an ELISA (enzyme-linked immunosorbent assay), chromatographic or enzymatic assay, or bioassay specific for the secreted product
5 can be used. If desired, secretory function of an implant can be monitored over time by collecting appropriate samples (e.g., serum) from the recipient and assaying them. If the recipient is a primate, microdialysis may be used.

10 The number of BAOs and BAO size should be sufficient to produce a therapeutic effect upon implantation is determined by the amount of biological activity required for the particular application. In the case of secretory cells releasing therapeutic
15 substances, standard dosage considerations and criteria known to the art are used to determine the amount of secretory substance required. Factors to be considered are discussed in Dionne, WO 92/19195.

 Implantation of the BAO is performed under
20 sterile conditions. Generally, the BAO is implanted at a site in the host which will allow appropriate delivery of the secreted product or function to the host and of nutrients to the encapsulated cells or tissue, and will also allow access to the BAO for
25 retrieval and/or replacement. The preferred host is a primate, most preferably a human.

 A number of different implantation sites are contemplated. These implantation sites include the central nervous system, including the brain, spinal
30 cord, and aqueous and vitreous humors of the eye. Preferred sites in the brain include the striatum, the cerebral cortex, subthalamic nuclei and nucleus Basalis

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of Meynert. Other preferred sites are the cerebrospinal fluid, most preferably the subarachnoid space and the lateral ventricles. This invention also contemplates implantation into the kidney subcapsular
5 site, and intraperitoneal and subcutaneous sites, or any other therapeutically beneficial site.

In order that this invention may be better understood, the following examples are set forth. These examples are for purposes of illustration only,
10 and are not to be construed as limiting the scope of this invention in any manner.

Examples

Construction of Polycistronic Expression Vectors

Construction of IgSP-POMC Fusion

15 The SmaI-SalI fragment containing the human POMC exon 3 was subcloned into pBS cloning vector (Stratagene). See Takahashi, supra; Cochet, supra. The resulting plasmid was named as pBS-hPOMC-027. See Fig. 1.

20 A PCR fragment was generated using two oligonucleotide primers, termed oCNTF-003 (SEQ ID NO: 1) and oIgSP-018, (SEQ ID NO: 2) and the pNUT plasmid containing the human CNTF gene. See Baetge et al., Proc. Natl. Acad. Sci. USA, 83, pp. 5454-58
25 (1986). Both primers oCNTF-003 and oIgSP-018, contain synthetic BamHI and SmaI restriction sites, respectively, at the 5' ends.

The 196 base pair (bp) PCR fragment was digested with restriction endonucleases BamHI and the
30 SmaI-isoschizomer XmaI, and electrophoresed through an

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1% SeaPlaque agarose. The 193 bp HindIII/XmaI DNA fragment was excised and purified using the FMC SpinBind DNA purification kit (FMC BioProducts, Rockland, ME).

5 pBS-hPOMC-027 was also digested with BamHI and XmaI and purified from 1% SeaPlaque agarose using the FMC SpinBind DNA purification kit (FMC BioProducts, Rockland, ME). The ligation mixture was transformed into E. coli DH5 α (Gibco BRL, Gaithersburg, MD).

10 Positive sub-clones were initially identified by the cracking gel procedure (Promega Protocols and Applications Guide, 1991). Minilysate DNA was then prepared using the FMC SpinBind DNA purification kit (FMC BioProducts, Rockland, ME) and subject to BamHI
15 and SmaI restriction digestions. The positive sub-clone was named as pBS-IgSP-hPOMC-028. See Fig. 1. The nucleotide sequence of the fusion junction in pBS-IgSP-hPOMC-028 was determined by the dideoxynucleotide sequence determination using the Sequenase kit (USBC,
20 Cleveland). The sequence of the IgSP-hPOMC fusion is shown in SEQ ID NO: 3.

Construction of IgSP-POMC Expression Vectors

The IgSP-hPOMC DNA fragment in pBS-IgSP-hPOMC-028 was subcloned into pcDNA3 (Invitrogen Corp.,
25 San Diego, CA) and pCEP4 (Invitrogen Corp., San Diego, CA) in sense and anti-sense orientations.

The NotI-SalI IgSP-hPOMC fragment from pBS-IgSP-hPOMC-028 was ligated with the NotI-XhoI digested pCEP4 resulting in the sense orientation clone named as
30 pCEP4-hPOMC-030. Fig. 2. The BamHI-SalI IgSP-hPOMC fragment from pBS-IgSP-hPOMC-028 was ligated with the

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BamHI-XhoI digested pCEP4 resulting in the anti-sense orientation clone named as pCEP4-hPOMC-031. Fig. 2. The insert orientation in pCEP4-hPOMC-030 and -031 was confirmed by BamHI, NotI, SalI and NotI/SalI
5 restriction digestions as well as by dideoxynucleotide sequence determination using the Sequenase kit (USBC, Cleveland).

The BamHI-SalI IgSP-hPOMC fragment from pBS-IgSP-hPOMC-028 was ligated with the BamHI-XhoI digested
10 pcDNA3 resulting in the sense orientation clone named as pcDNA3-hPOMC-034. Fig. 2. The NotI-HindIII IgSP-hPOMC fragment from pBS-IgSP-hPOMC-028 was ligated with the NotI-HindIII digested pcDNA3 resulting in the anti-sense orientation clone named as pcDNA3-hPOMC-035.

15 Fig. 2. Restriction digestion using SmaI, BamHI, EcoRI, and BamHI/EcoRI was used to confirm the insert orientation in pcDNA3-hPOMC-034, whereas HindIII, NotI and SalI were used for pcDNA3-hPOMC-035.

Construction of ACTH Deleted IgSP-POMC

20 The ACTH coding region in the POMC gene in pBS-IgSP-hPOMC-028 was deleted. pBS-IgSP-hPOMC-028 was first digested with XmaI restriction enzyme and treated with pfu DNA polymerase (Promega, Madison, WI). The XmaI-pfu DNA polymerase treated pBS-IgSP-hPOMC-028 was
25 then digested with StuI restriction enzyme and purified from 1% SeaPlaque agarose using the FMC SpinBind DNA purification kit (FMC BioProducts, Rockland, ME). The self-ligation mixture was transformed into E. coli DH5 α (Gibco BRL, Gaithersburg, MD). Positive sub-clones
30 were identified by BamHI/HindIII restriction digestion and named as pBS-IgSP-hPOMC Δ ACTH-029. See Fig. 1. The

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nucleotide sequence of the ACTH deletion region in pBS-IgSP-hPOMC- Δ ACTH-029 was confirmed by the dideoxynucleotide sequence determination. The sequence of the IgSP-hPOMC- Δ ACTH fusion is shown in SEQ ID
5 NO: 4.

Construction of ACTH Deleted IgSP-POMC Expression Vectors

The IgSP-hPOMC- Δ ACTH DNA fragment in pBS-IgSP-hPOMC- Δ ACTH-029 was subcloned into pcDNA3
10 (Invitrogen Corp., San Diego, CA) and pCEP4 (Invitrogen Corp., San Diego, CA) in sense and anti-sense orientations. The NotI-SalI IgSP-hPOMC- Δ ACTH fragment from pBS-IgSP-hPOMC- Δ ACTH-029 was ligated with the NotI-XhoI digested pCEP4 resulting in the sense
15 orientation clone named as pCEP4-hPOMC- Δ ACTH-032 (Fig. 3). The BamHI-SalI IgSP-hPOMC- Δ ACTH fragment from pBS-IgSP-hPOMC- Δ ACTH-029 was ligated with the BamHI-XhoI digested pCEP4 resulting in the anti-sense orientation clone named as pCEP4-hPOMC- Δ ACTH-033
20 (Fig. 3). The insert orientation in pCEP4-hPOMC- Δ ACTH-032 and -033 was confirmed by BamHI and EcoRI restriction digestions as well as by dideoxynucleotide sequence determination using the Sequenase kit (USBC, Cleveland).

25 The BamHI-SalI IgSP-hPOMC- Δ ACTH fragment from pBS-IgSP-hPOMC- Δ ACTH-029 was ligated with the BamHI-XhoI digested pcDNA3 resulting in the sense orientation clone named as pcDNA3-hPOMC- Δ ACTH-036 (Fig. 3). The NotI-HindIII IgSP-hPOMC- Δ ACTH fragment from pBS-IgSP-
30 hPOMC- Δ ACTH-029 was ligated with the NotI-HindIII

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digested pcDNA3 resulting in the anti-sense orientation clone named as pcDNA3-hPOMC- Δ ACTH-037 (Fig. 3).

Restriction digestion using PvuII and EcoRI was used to confirm the insert orientation in pcDNA3-hPOMC- Δ ACTH-036, whereas SalI and EcoRI were used for pcDNA3-hPOMC- Δ ACTH-037.

Cloning of Full Length and Truncated TH cDNA

Total RNA from PC12 cells was prepared using the guanidinium thiocyanate-based TRI reagent (Molecular Research Center, Inc., Cincinnati, OH). Five hundred ng of PC12 total RNA was reverse transcribed at 42°C for 30 minutes in a 20 μ l reaction volume containing 10 mM Tris.HCl (pH 8.3), 50 mM KCl, 4 mM of each dNTP, 5 mM MgCl₂, 1.25 μ M oligo (dT) 15-mer, 1.25 μ M random hexamers, 31 units of RNase Guard RNase Inhibitor (Pharmacia, Sweden) and 200 units of SuperScript II reverse transcriptase (Gibco BRL, Gaithersburg, MD). Two micro-liters of the above reverse transcribed cDNA was added to a 25 μ l PCR reaction mixture containing 10 mM Tris.HCl (pH 8.3), 50 mM KCl, 800 of each nM dNTP, 2 mM MgCl₂, 400 nM of primers #1 and #2, and 2.5 units of Thermus aquaticus (Taq) DNA polymerase (Boehringer Mannheim, Germany).

To generate the full length TH cDNA, oligonucleotide primers orTH-052 (SEQ ID NO: 5) and orTH-053 (SEQ ID NO: 6) were used. For the truncated TH, primers orTH-054 (SEQ ID NO: 7) and orTH-053 (SEQ ID NO: 6) were used instead. These oligonucleotides were constructed based on published TH sequence information in Grima et al., Nature, 326, pp. 707-11 (1987); US patent 5,300,436, and Daubner, supra.

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Primers orTH-052 (SEQ ID NO: 5) and orTH-054 (SEQ ID NO: 7) have synthetic HindIII restriction site at the 5' end where orTH-053 has BamHI at the 5' end. The PCR reaction mixtures were subject to 30

5 amplification cycles consisted of: denaturation, 94°C 30 seconds (first cycle 2 minutes); annealing, 50°C 1 minute; and extension, 72°C 3.5 minutes (last cycle 5 minutes). The 1537 bp full length and 1087 bp truncated rat TH PCR fragments were digested with

10 restriction endonucleases BamHI and HindIII and resolved on an 1% SeaPlaque agarose gel. The 1531-bp and 1081-bp HindIII/BamHI DNA fragments were excised and purified using the FMC SpinBind DNA purification kit (FMC BioProducts, Rockland, ME).

15 pcDNA3 expression vector was also digested with BamHI and HindIII and purified from 1% SeaPlaque agarose using the FMC SpinBind DNA purification kit (FMC BioProducts, Rockland, ME). The ligation mixture was transformed into E.coli DH5α (Gibco BRL,

20 Gaithersburg, MD).

Cracking gel procedure (Promega Protocols and Applications Guide, 1991) was used to screen out the positive sub-clones. The identity of the correct clones was further verified by BamHI/HindIII double

25 digestion.

The positive sub-clones for the full-length and truncated rat TH in pcDNA3 were named as pcDNA3-rTH-044 (Fig. 4) and pcDNA3-rTHΔ-045 (Fig. 4), respectively. The nucleotide sequence of both full-

30 length and truncated rat TH PCR clones was determined by the dideoxynucleotide sequence determination using

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the Sequenase kit (USBC, Cleveland). The sequence of the rTHA construct is shown in SEQ ID NO: 16.

To optimize the translation efficiency of the truncated rat TH, oligonucleotide primer orTH-078 (SEQ ID NO: 8) was designed so that the consensus Kozak sequence is immediate up stream to the start codon ATG. pcDNA3-rTHA-45 was used as the template in a 50 µl PCR reaction mixture with reagent composition identical to the one described above with the exception that the oligonucleotide primers were replaced with orTH-078 (SEQ ID NO: 8) and orTH-053 (SEQ ID NO: 6). The 1097 bp PCR product was cloned into pcDNA3 in the same manner as described above. The resulting sub-clone was named pcDNA3-rTHAKS-75 (Fig 4). The sequence of the rTHAKS construct is shown in SEQ ID NO: 17.

Construction of rTH-IRES-bDBH Fusion Gene

Recombinant PCR methodology was used to generate the rTH-IRES-bDBH fusion gene.

Oligonucleotides oIRES-057 (SEQ ID NO: 9) and obDBH-065 (SEQ ID NO: 10) are specific for IRES and bDBH gene sequences, respectively, and contain synthetic BamHI and NotI restriction sites at the 5' end, respectively. Oligonucleotides oIRES-bDBH-064 (SEQ ID NO: 11) and oIRES-bDBH-066 (SEQ ID NO: 12) are complementary to each other. Furthermore, oligonucleotide primer oIRES-bDBH-064 (SEQ ID NO: 11) has its 5' 16 nucleotides identical to the IRES sequence and its 3' 18 nucleotides identical to the bDBH sequence; and vice versa for oIRES-bDBH-066 (SEQ ID NO: 12).

Two first PCR reactions were carried out using oligonucleotide pairs oIRES-057/oIRES-bDBH-066

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and oIRES-bDBH-064/obDBH-065 on templates pCTI-001
(with an insert containing the IRES sequence shown in
SEQ ID NO: 30) and pBS-bDBH-006 (containing the bovine
DBH gene cloned from bovine adrenal chromaffin cells,
5 Lamoroux et al., EMBO J., 6, pp. 3931-37 (1987))
plasmids, respectively. One hundred ng of template DNA
was added to a 50 µl PCR reaction mixture containing
10 mM Tris.HCl (pH 8.3), 50 mM KCl, 800 of each nM
dNTP, 2 mM MgCl₂, 400 nM of primers #1 and #2, and 2.5
10 units of Thermus aquaticus (Taq) DNA polymerase
(Boehringer Mannheim, German).

The PCR reaction mixtures were subject to 30
amplification cycles consisted of: denaturation, 94 °C
for 30 seconds (first cycle 2 minutes); annealing,
15 50 °C 1 minute; and extension, 72 °C 30 seconds (last
cycle 5 minutes). The PCR products were resolved on 1%
TrivieGel 500 (TrivieGen). Two agarose plugs
containing each one of the first PCR products were
transfer to a tube containing 50 µl of PCR reaction
20 mixtures identical to the one described above with the
exception that the oligonucleotides oIRES-057 and
obDBH-065 were used.

The second PCR reaction was subject to 30
amplification cycles consisted of: denaturation, 94 °C
25 for 30 seconds (first cycle 2 minutes); annealing,
60 °C 30 seconds (second to fourth cycles 37 °C 2
minutes); and extension, 72 °C 30 seconds (last cycle 2
minutes). The 2407 bp IRES-bDBH fusion PCR product and
the cloning vector pcDNA3-rTHA-45 were digested with
30 BamHI and NotI restriction enzymes and subsequently
purified from 1% SeaPlaque agarose gel using the FMC

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SpinBind DNA purification kit (FMC BioProducts, Rockland, ME).

The ligation of IRES-bDBH/BamHI/NotI and pcDNA3-rTHA-045/BamHI/NotI would generate a rTHA-IRES-
5 bDBH expression vector named as pcDNA3-rTHA-IRES-bDBH-066 (Fig. 5) whereas that of IRES-bDBH/BamHI/NotI and pcDNA3-rTHAKS-075/BamHI/NotI would generate a rTHAKS-IRES-bDBH expression vector, named as pcDNA3-rTHAKS-IRES-bDBH-076 (Fig. 5), where the start codon ATG in
10 rTHA is preceded with a consensus Kozak sequence. The sequence of the rTHA-IRES-bDBH construct is shown in SEQ ID NO: 18. The sequence of the rTHAKS-IRES-bDBH construct is shown in SEQ ID NO: 19. The ligation mixture was transformed into DH5 α (Gibco BRL,
15 Gaithersburg, MD). The positive clones were identified by the cracking gel procedure (Promega, Madison, WI) and restriction digestions using HindIII, BamHI, HindIII/BamHI, SmaI and NotI.

The 4114 bp NruI-XhoI fragment containing the
20 CMV promoter-rTHAKS-IRES-bDBH was excised out of pcDNA3-rTHAKS-IRES-bDBH-076 and subcloned into pZeoSV cloning vector (Invitrogen Corp., San Diego, CA) digested with ScaI and XhoI in the multiple cloning site. The resulting expression vector was named as
25 pZeo-Pcmv-rTHAKS-IRES-bDBH-088 (Fig. 6).

Construction of IgSP-hPOMC ACTH-rTHD-IRES-bDBH Fusion Gene

The 4100 bp NruI-NotI fragment containing the
CMV promoter, rTHD-IRES-bDBH fusion gene, and BGH
30 polyadenylation sequence was excised out of pcDNA3-

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rTHA-IRES-bDBH-066 and subcloned into the pBS
(Stratagene, La Jolla, CA) cloning vector.

The resulting plasmid pBS-Pcmv-rTHA-IRES-
bDBH-067 (Fig. 7) was used as the intermediary
5 construct to which the recombinant PCR IgSP-hPOMCDACTH-
IRES fragment would be inserted.

Oligonucleotide oIgSP-068 (SEQ ID NO: 13),
containing a synthetic EcoRV restriction site, is
specific for the IgSP sequence.

10 Oligonucleotide primer orTHA-073 (SEQ ID
NO: 14) is specific for the rTHA sequence and contains
an endogenous SmaI restriction site.

Oligonucleotide primers ohPOMC-IRES-069 (SEQ
ID NO: 15) and ohPOMC-IRES-070 (SEQ ID NO: 20) are
15 complementary to each other. Furthermore,
oligonucleotide primer ohPOMC-IRES-069 has its 5', 18
nucleotides identical to the hPOMC sequence and its 3'
12 nucleotides identical to the IRES sequence; and vice
versa for ohPOMC-IRES-070.

20 Oligonucleotide primers oIRES-rTHA-071 (SEQ
ID NO: 21) and oIRES-rTHA-072 (SEQ ID NO: 22) are
complementary to each other. In addition,
oligonucleotide primer oIRES-rTHA-071 has its 5' 15
nucleotides identical to the rTHA sequence and its 3'
25 18 nucleotide identical to the IRES sequence; and vice
versa for oIRES-rTHA-072.

Three sets of first PCR reactions were
carried out.

PCR reaction A: template pBS-IgSP-hPOMCDACTH-029,
30 oligonucleotides oTgSP-068/ohPOMC-IRES-069;

PCR reaction B: template pCTI-001,
oligonucleotides ohPOMC-IRES-070/oIRES-rTHA-071; and

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PCR reaction C: template pCDNA3-rTHA-045,
oligonucleotides orIRES-rTHA-072/orTHA-073.

The three sets of first PCR reactions were
carried in 50 µl PCR reaction mixture containing 100 ng
5 of template DNA, 10 mM Tris. HCl (pH 8.3), 50 mM KCl,
800 of each nM dNTP, 2 mM MgCl₂, 400nM of primers #1
and #2, and 2.5 units of Thermus aquaticus (Taq) DNA
polymerase (Boehringer Mannheim, Germany).

The PCR reaction mixtures were subject to 30
10 amplification cycles consisted of: denaturation, 94 °C
for 30 seconds (first cycle 2 minutes); annealing,
50 °C 1 minute; and extension, 72 °C 30 seconds (last
cycle 5 minutes).

The PCR products were resolved on 1%
15 TrivieGel 500 (TrivieGen). Two agarose plugs
containing each one of the PCR products from PCR
reactions B and C were transferred to a tube containing
50 µl of PCR reaction mixtures identical to the one
described above with the exception that the
20 oligonucleotides ohPOMC-IRES-070 and orTHA-073 were
used.

The second PCR reaction was subject to 30
amplification cycles consisted of: denaturation, 94 °C
for 30 seconds (first cycle 2 minutes); annealing,
25 60 °C 30 seconds (second to fourth cycles 37 °C 2
minutes); and extension, 72 °C 30 seconds (last cycle 2
minutes).

The PCR products were treated as described
above. Agarose plugs containing the PCR products from
30 the second PCR reaction and the PCR reaction A were
combined and subjected to a third PCR amplification
using oIgSP-068/rTHA-073. The 1203 bp IgSP-hPOMC-IRES-

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rTHA fusion PCR product and the cloning vector pBS-Pcmv-rTHA-IRES-bDBH-067 were digested with EcoRV and XmaI restriction enzymes and subsequently purified from 1% SeaPlaque agarose gel using the FMC SpinBind DNA
5 purification kit (FMC BioProducts, Rockland, ME). The ligation mixture was transformed into DH5 α (Gibco BRL, Gaithersburg, MD).

The positive clones were identified by the cracking gel procedure (Promega, Madison, WI) and
10 restriction digestions using EcoRI, KpnI and NotI. The resulting clone was named as pBS-IgSP-hPOMCAACTH-IRES-rTHA-IRES-bDBH-068. Fig. 8. The sequence of this construct is shown in SEQ ID NO: 23.

15 Construction of IgSP-hPOMCAACTH-IRES-rTHA-IRES-bDBH Expression Vectors

The 4491 bp NotI fragment containing the IgSP-hPOMCAACTH-IRES-rTHA-IRES-bDBH gene was excised out of the pBS-IgSP-hPOMCAACTH-IRES-rTHA-IRES-bDBH-068 and subcloned into the pcDNA3 (Invitrogen Corp., San
20 Diego, CA) at the NotI site in the multiple cloning site. Restriction digestion using NotI and SmaI confirmed that the IgSP-hPOMCAACTH-IRES-rTHA-IRES-bDBH gene was inserted in the sense orientation resulting in pcDNA3-IgSP-hPOMCAACTH-IRES-rTHA-IRES-bDBH-069. See
25 Fig. 9.

Construction of IgSP-hPOMCAACTH-IRES-rTHA-IRES-bDBH-IRES-Zeocine Expression Vector

Recombinant PCR methodology was used to generate the IRES-Zeocine fusion gene.
30 Oligonucleotides oIRES-074 (SEQ ID NO: 24) and oZeocin-

- 40 -

077 (SEQ ID NO: 25) are specific for IRES and Zeocin gene sequences, respectively, and contain synthetic NotI and XhoI restriction sites at the 5' end, respectively. Oligonucleotides oIRES-Zeocin-075 (SEQ ID NO: 26) and oIRES-Zeocin-076 (SEQ ID NO: 27) are complementary to each other. Furthermore, oligonucleotide oIRES-Zeocin-075 has its 5'15 nucleotides identical to the Zeocin sequence and its 3' 18 nucleotides identical to the IRES sequence; and vice versa for oIRES-Zeocin-076.

Two first PCR reactions were carried out using oligonucleotide pairs oIRES-074/oIRES-Zeocin-075 and oIRES-Zeocin-076/oZeocin-075 on templates pCTI-001 and pZeoSV (Invitrogen Corp., San Diego, CA) plasmids, respectively.

One hundred ng of template DNA was added to a 50 µl PCR reaction mixture containing 10mM Tris.HCl (pH 8.3), 50 mM KCl, 800 of each nM dNTP, 2 mM MgCl₂, 400 nM of primers #1 and #2, and 2.5 units of Thermus 20 aquaticus (Taq) DNA polymerase (Boehringer Mannheim, Germany).

The PCR reaction mixtures were subject to 30 amplification cycles consisted of: denaturation, 94 °C for 30 seconds (first cycle 2 minutes); annealing, 25 50 °C 1 minute; and extension, 72 °C 30 seconds (last cycle 5 minutes).

The PCR products were resolved on 1% TrivieGel 500 (TrivieGen). Two agarose plugs containing each one of the first PCR products were 30 transfer to a tube containing 50 µl of PCR reaction mixtures identical to the one described above with the

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exception that the oligonucleotides oIRES-074 and oZeocin-077 were used.

The second PCR reaction was subject to 30 amplification cycles consisted of: denaturation, 94 °C for 30 seconds (first cycle 2 minutes); annealing, 50 °C 30 seconds (second to fourth cycles 37 °C 2 minutes); and extension, 72 °C 30 seconds (last cycle 2 minutes).

The 974 bp IRES-Zeocin fusion PCR product and the cloning vector pcDNA3 were digested with NotI and XhoI restriction enzymes and subsequently purified from 1% SeaPlaque agarose gel using the FMC SpinBind DNA purification kit (FMC BioProducts, Rockland, ME).

The ligation of IRES-Zeocin/NotI/XhoI and pcDNA3/NotI/XhoI would generate an intermediate cloning vector named as pcDNA3-IRES-Zeocin-072. Fig. 10.

The positive clones were identified by the cracking gel procedure (Promega, Madison, WI) and restriction digestions using HindIII, SmaI, XhoI, NotI and NotI/XhoI.

To generate the final IgSP-hPOMCDACTH-IRES-rTHD-IRES-bDBH-IRES-Zeocine Expression Vector, a 4491 bp NotI fragment containing the IgSP-hPOMCAACTH-IRES-rTHA-IRES-bDBH gene was excised out of the pBS-IgSP-hPOMCAACTH-IRES-rTHA-IRES-bDBH-068 (Fig. 8; SEQ ID NO: 23) and subcloned in to the pcDNA3-IRES-Zeocin-072 (Fig. 10) at the NotI site in the multiple cloning site.

Restriction digestion using NotI and SmaI confirmed that the IgSP-hPOMCAACTH-IRES-rTHA-IRES-bDBH gene was inserted in the sense orientation resulting in pcDNA3-IgSP-hPOMCAACTH-IRES-rTHA-IRES-bDBH-IRES-Zeocin-

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073. The sequence of this construct is shown in SEQ ID NO: 28. Fig. 11.

Construction of ProA+KS Fusion

A construct containing the coding region of the human pro-enkephalin A gene with the consensus Kozak sequence immediately upstream to the start codon ATG. The sequence of this construct is shown in SEQ ID NO: 29.

Construction of hProA+KS Expression Vector

The HindIII/BamHI fragment containing the hProA+KS fusion was ligated into BamHI and Hind III digested pcDNA3 expression vector substantially as described above. After screening as described above, a positive sub-clone was named pcDNA3-hProA+KS-091. Fig. 12. Construction of the pBS-CMV Pro A vector is detailed in Mothis, J. and Lindberg, I., Endocrinology, 131, pp. 2287-96 (1992).

Transformation of Cells

RIN and AtT-20 cells were transformed as follows.

The RINa and AtT-20 based cell lines were grown in DMEM (Gibco) with 10% fetal bovine serum and pen-strep-fungizone (Gibco) base media. The cells were plated out in P100 petri dishes (750,000 cells/dish) in 10 ml of base media. 18-24 hours later, the cells were transfected using calcium phosphate method with a kit made by Stratagene (San Diego, CA). A 10 µg amount of the plasmid vector DNA was diluted in 450 µl of deionized sterile water. Then, 50 µl of a 10x buffer

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(solution #1) was added to the plasmid DNA. A 500 μ l amount of solution #2 was immediately added to the DNA containing solution and mixed gently. This was incubated at room temperature for 20 minutes and then
5 the 1.0 ml solution was added to the cells in the petri dish. The cells were incubated overnight and 18-24 hours later the cells were washed 2x with Hanks balanced salt solution without calcium and magnesium. Then, the cells were cultured in base media + selection
10 drugs. The cells were selected in either 600 μ g/ml geneticin (Gibco) or 400 μ g/ml hygromycin (Boehringer Mannheim) or 500 μ g/ml Zeocin (In Vitrogen, San Diego, CA). Cells were sequentially transfected and selected to obtain the final cell line.

15 The RiNa cells were transfected with plasmid pCEP4-hPOMC-030 containing the POMC gene. This is a hygromycin resistant vector. The cells were also transformed with plasmid pcDNA3-hProA+KS-091. This is a geneticin resistant vector. Finally, the cells were
20 transfected with plasmid pZeo-PCMV-rTHAKS-IRES-bDBH-088 which conferred Zeocin resistance.

The AtT-20 cells were transfected with plasmid pBS-CMV-ProA and pCEP4-POMC- Δ ACTH-32 which conferred geneticin and hygromycin resistance,
25 respectively. Finally, the cells were transfected with plasmid pZeo-Pcmv-rTHAKS-IRES-bDBH-088.

We have tested a number of media for cell growth. Surprisingly we have found that in certain serum-free medias, the above cell lines have enhanced
30 neurotransmitter output, compared to serum-containing media. We prefer CHO-Ultra (Biowhitaker) for the

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growth of AtT-20 cells, and Ultra-Culture (Biowhitaker) for the growth of RINa cells.

Output of various analgesics from one transformed RINa cell line (RINa/ProA/P030/P088) is shown in Table 2. All values represent unstimulated cells. Output of β -endorphin and met-enkephalin is in pg/ 10^6 cells/hr. β -endorphin and met-enkephalin were measured by radioimmunoassay using Incstar kits (Stillwater, Minnesota). Catecholamine output is in pmoles/ 10^6 cells/hr. The numbers in parentheses represent values from cells that were preincubated 18 hours with 100 μ M tetrahydrobiopterin. Catecholamines were measured by high performance liquid chromatography as described in Lavoie et al., "Two PC12 pheochromocytoma lines sealed in hollow fiber-based capsules tonically release l-dopa in vitro", Cell transplantation, 2, pp. 163-73 (1993). GABA output from these RINa cells was 28 ng/ 10^6 cells/hrs.

Table 2

20	<u>Cell Line</u>	<u>Endogenous Analgesic Substances</u>	<u>β-endorphin</u>	<u>Met-enk</u>	<u>DA E</u>
	RIN a/ ProA/ POMC/	β -endorphin GABA	22	17	3 0 (6) (2)
25	TH-IRES-D β H				

There are encrypted enkephalin fragments which are not fully processed from the pro-enkephalin precursor molecule. These encrypted enkephalins have opioid receptor binding activity. We digested these encrypted enkephalins to measure opioid activity. The trypsin digest protocol is as follows. A 2 μ g/ml trypsin (Worthington #34E470) solution is added to media

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samples on ice. Samples are vortexed, then incubated for 20 minutes in a 37°C waterbath. After the 20 minute digest, samples are returned to ice and 100 ng/ml carboxypeptidase B (Sigma #C-7011) is added.

5 Samples are mixed by vortexing, and returned to the 37°C waterbath for 15 minutes. Samples are placed on ice once more and 10 ug/ml trypsin inhibitor is added. At this stage, samples are either extracted for met-enkephalin or immediately frozen for future extraction.

10 This results in the full enzymatic cleavage to free all met-enkephalin from the longer encrypted fragments. A met-enkephalin radioimmunoassay of the digested sample gives total met-enkephalin from the supernatant. The transformed RINa cells appear to have greater than 5

15 fold more encrypted enkephalins compared to fully processed met-enkephalin.

Fiber capsule formation and characteristics

Hollow fibers are spun from a 12.5-13.5% poly(acrylonitrile vinylchloride) solution by a wet

20 spinning technique. Cabasso, Hollow Fiber Membranes, vol. 12, Kirk-Othmer Encyclopedia of Chemical Technology, Wiley, New York, 3rd Ed. pp. 492-517 (1980), United States patent 5,158,881, incorporated herein by reference.

25 The resulting membrane fibers may either be double skinned or single skinned PAN/PVC fibers. In order to make implantable capsules, lengths of fiber are first cut into 5 cm long segments and the distal extremity of each segment sealed with an acrylic glue.

30 Encapsulation hub assemblies are prepared by providing lengths of the membrane described above, sealing one

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end of the fiber with a single drop of LCM 24 (Light curable acrylate glue, available from ICI), curing the glue with blue light, and repeating the step with a second drop. The opposite end is previously attached
5 to a frangible necked hub assembly, having a silicone septum through which the cell solution may be introduced. The fiber is glued to the hub assembly by applying LCM 22 to the outer diameter of the hub assembly, pulling the fiber up over it, and curing with
10 blue light. The hub/fiber assemblies are placed in sterilization bags and are ETO sterilized.

Following sterilization with ethylene oxide and outgassing, the fibers are deglycerinated by ultrafiltering first 70% EtOH, and then HEPES buffered
15 saline solution through the walls of the fiber under vacuum.

Preparation and Encapsulation of Transformed Cells

The transformed cells are prepared and encapsulated as follows:

20 A matrix solution is prepared using a commercially available alginate, collagen or other suitable matrix material. The cell solution was diluted in the ratio of two parts matrix solution to one part cell solution containing the transformed cells
25 described above. We prefer Vitrogen (Celtix, Santa Clara) as a matrix for AtT-20 cells.

We prefer Organogen (Organogenesis, Canton, MA) as a matrix for RINa cells. The RINa based cells are prepared for encapsulation by the following method.
30 The cells are grown in base media of DMEM + 10% fetal bovine serum during the proliferation phase. These

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cells can be removed from the tissue culture flasks by two washes in Hanks balanced salt solution without calcium and magnesium. Then the cells are incubated in 0.25% trypsin + EDTA for 1 minute. This is removed and
5 the cells are rinsed free of the flask using Hanks balanced salt solution without calcium and magnesium solution. The cells are placed in 10 mls of base media and centrifuged at 100 x g for 2 minutes. The cells are resuspended in 10 mls of the preferred serum free
10 media (Ultra culture, Biowhitaker, Walkersville, MD). Surprisingly, the RINa cells secrete more analgesic substances when cultured in this serum free media relative to serum containing base media.

The cells are centrifuged at 100 g twice in
15 the preferred serum free media before the cells are concentrated 1:1 with the preferred Organogen matrix. Organogen is a 1% bovine tendon collagen obtained as a sterile solution. 8 parts of this solution are mixed with 1 part 10X DPBS. 0.5 N sodium hydroxide is added
20 until physiological pH is attained (approximately 250 μ ls).

The final concentration of the cell + matrix solution used for encapsulation can range from 20,000 - 50,000 cells/ μ l. The cells are counted in a standard
25 manner on a hemocytometer.

The cell/matrix suspension is placed in a 1 ml syringe. A Hamilton 1800 Series 50 microliter syringe is set for a 15 microliter air bubble, is inserted into a 1 ml syringe containing the cell
30 solution and 30 microliters are drawn up. The cell solution is injected through the silicone seal of the hub/fiber assembly into the lumen of a modacrylic

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hollow fiber membrane with a molecular weight cutoff of approximately 50,000-100,000 daltons. Ultrafiltration should be observed along the entire length of the fiber. After one minute, the hub is snapped off the sub-hub, exposing a fresh surface, unwet by cell solution. A single drop of LCM 24 is applied and the adhesive cured with blue light. The device is placed first in HEPES buffered NaCl solution and then in CaCl_2 solution for five minutes to cross-link the alginate.

10 Each implant is about 5 cm long, 1 mm in diameter, and contained approximately 2.5 million cells.

After the devices are filled and sealed, a silicone tether (Speciality Silicone Fabrication, Paso Robles, CA) (ID: 0.69, OD: 1.25) is then placed over the proximal end of the fiber. A radiopaque titanium plug is inserted in the lumen of the silicone tether to act as a radiographic marker. The devices are then placed in 100 mm tissue culture dishes in 1.5 ml PC-1 medium, and stored at 37°C, in a 5% CO_2 incubator for

15 in vitro analysis and for storage until implantation.

The encapsulated cells are then implanted into the human sub-arachnoid space as follows:

Surgical Procedure

After establishing IV access and administering prophylactic antibiotics (cefazolin sodium, 1 gram IV), the patient is positioned on the operating table, generally in either the lateral decubitus or genu-pectoral position, with the lumbar spine flexed anteriorly. The operative field is

25 sterily prepared and draped exposing the midline dorsal lumbar region from the levels of S-1 to L-1, and

30

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allowing for intraoperative imaging of the lumbar spine with C-arm fluoroscopy. Local infiltration with 1.0% lidocaine is used to establish anesthesia of the skin as well as the periosteum and other deep connective
5 tissue structures down to and including the ligamentum flavum.

A 3-5 cm skin incision is made in the parasagittal plane 1-2 cm to the right or left of the midline and is continued down to the lumbodorsal
10 fascia using electrocautery for hemostasis. Using traditional bony landmarks including the iliac crests and the lumbar spinous processes, as well as fluoroscopic guidance, and 18 gauge Touhy needle is introduced into the subarachnoid space between L-3 and
15 L-4 via an oblique paramedian approach. The needle is directed so that it enters the space at a shallow, superiorly directed angle that is no greater than 30-35° with respect to the spinal cord in either the sagittal or transverse plane. Appropriate position of
20 the tip of the needle is confirmed by withdrawal of several ml of cerebrospinal fluid (CSF) for preimplantation catecholamine, enkephalin, glucose, and protein levels and cell counts.

The Touhy needle hub is reexamined to confirm
25 that the opening at the tip is oriented superiorly (opening direction is marked by the indexing notch for the obturator on the needle hub), and the guide wire is passed down the lumen of the needle until it extends 4-5 cm into the subarachnoid space (determined by
30 premeasuring). Care is taken during passage of the wire that there is not resistance to advancement of the wire out of the needle and that the patient does not

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complain of significant neurogenic symptoms, either of which observations might indicate misdirection of the guide wire and possible impending nerve root or spinal cord injury.

5 After the guide wire appears to be appropriately placed in the subarachnoid space, the Touhy needle is separately withdrawn and removed from the wire. The position of the wire in the midline of the spinal canal, anterior to the expected location of
10 the caud equina, and without kinks or unexplainable bends is then confirmed with fluoroscopy. After removal of the Touhy needle the guide wire should be able to be moved freely into and out of the space with only very slight resistance due to the rough surface of
15 the wire running through the dense and fibrous ligamentum flavum.

 The 7 French dilator is then placed over the guide wire and the wire is used to direct the dilator as it is gently but firmly pushed through the fascia,
20 paraspinous muscle, and ligamentum flavum, following the track of the wire toward the subarachnoid space. Advancement of the 7 French dilator is stopped and the dilator removed from the wire as soon as a loss of resistance is detected after passing the ligamentum
25 flavum. This is done in order to avoid advancing and manipulating this relatively rigid dilator within the subarachnoid space to any significant degree.

 After the wire track is "overdilated" by the 7 French dilator, the 6 French dilator and cannula
30 sheath are assembled and placed over the guide wire. The 6 French dilator and cannula are advanced carefully into the subarachnoid space until the opening tip of

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the cannula is positioned 7 cm within the space. As with the 7 French dilator, the assembled 6 French dilator and cannula are directed by the wire within the lumen of the dilator. Position within the subarachnoid space is determined by premeasuring the device and is grossly confirmed by fluoroscopy. Great care is taken with manipulation of the dilators and cannula within the subarachnoid space to avoid misdirection and possible neurologic injury.

10 When appropriate positioning of the cannula is assured, the guide wire and the 6 French dilator are gently removed from the lumen of the cannula in sequence. Depending on the patient's position on the operating table, CSF flow through the cannula at this point should be noticeable and may be very brisk, requiring capping the cannula or very prompt placement of the capsule implant in order to prevent excessive CSF.

 The encapsulated (transformed cells) is provided in a sterile, double envelope container, bathed in transport medium, and fully assembled including a tubular silicone tether. Prior to implantation through the cannula and into the subarachnoid space, the capsule is transferred to the insertion kit tray where it is positioned in a location that allowed the capsule to be maintained in transport medium while it is grossly examined for damage or major defects, and while the silicone tether is trimmed, adjusting its length to the pusher and removing the hemaclip™ that plugs its external end.

 The tether portion of the capsule is mounted onto the stainless steel pusher by inserting the small

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diameter wire portion of the pusher as the membrane portion of the device is carefully introduced into the cannula. The capsule is advanced until the tip of the membrane reaches a point that is 2-10 mm within the cranial tip of the cannula in the subarachnoid space. This placement is achieved by premeasuring the cannula and the capsule-tether-pusher assembly, and it assures that the membrane portion of the capsule is protected by the cannula for the entire time that it is being advanced into position.

After the capsule is positioned within the cannula, the pusher is used to hold the capsule in position (without advancing or withdrawing) in the subarachnoid space while the cannula is completely withdrawn from over the capsule and pusher. The pusher is then removed from the capsule by sliding its wire portion out of the silicone tether. Using this method the final placement of the capsule is such that the 5 cm long membrane portion of the device lay entirely within the CSF containing subarachnoid space ventral to the cauda equina. It is anchored at its caudal end by a roughly 1-2 cm length of silicone tether that runs within the subarachnoid space before the tether exits through the dura and ligamentum flavum. The tether continues externally from this level through the paraspinous muscle and emerges from the lumbodorsal fascia leaving generally 10-12 cm of free tether material that is available for securing the device.

CSF leakage is minimized by injecting fibrin glue (Tissel®) into the track occupied by the tether in the paraspinous muscle, and by firmly closing the superficial fascial opening of the track with a purse-

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string suture. The free end of the tether is then anchored with non-absorbable suture and completely covered with a 2 layer closure of the skin and subcutaneous tissue.

- 5 The patient is then transferred to the neurosurgical recovery area and kept at strict bed rest, recumbent, for 24 hours postoperatively. Antibiotic prophylaxis is also continued for 24 hours following the implantation procedure.

10 Sequences

The following is a summary of the sequences set forth in the Sequence Listing:

- SEQ ID NO:1 -- DNA sequence of oligo oCNTF-003
 SEQ ID NO:2 -- DNA sequence of oligo oIgSP-018
 15 SEQ ID NO:3 -- DNA sequence of IgSP-hPOMC fusion
 SEQ ID NO:4 -- DNA sequence of IgSP-hPOMC- Δ ACTH fusion
 SEQ ID NO:5 -- DNA sequence of oligo orTH-052
 SEQ ID NO:6 -- DNA sequence of oligo orTH-053
 SEQ ID NO:7 -- DNA sequence of oligo orTH-054
 20 SEQ ID NO:8 -- DNA sequence of oligo orTH-078
 SEQ ID NO:9 -- DNA sequence of oligo oIRES-057
 SEQ ID NO:10 -- DNA sequence of oligo obDBH-065
 SEQ ID NO:11 -- DNA sequence of oligo oIRES-bDBH-064
 SEQ ID NO:12 -- DNA sequence of oligo oIRES-bDBH-066
 25 SEQ ID NO:13 -- DNA sequence of oligo oIRE-068
 SEQ ID NO:14 -- DNA sequence of oligo orTHA-073
 SEQ ID NO:15 -- DNA sequence of oligo ohPOMC-IRES-069
 SEQ ID NO:16 -- DNA sequence of rTHA1-155
 SEQ ID NO:17 -- DNA sequence of rTHA+KS
 30 SEQ ID NO:18 -- DNA sequence of rTHA-IRES-bDBH
 SEQ ID NO:19 -- DNA sequence of rTHAKS-IRES-bDBH

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- SEQ ID NO:20 -- DNA sequence of oligo ohPOMC-IRES-070
 SEQ ID NO:21 -- DNA sequence of oligo oIRES-rTHA-071
 SEQ ID NO:22 -- DNA sequence of oligo orIRES-rTHA-072
 SEQ ID NO:23 -- DNA sequence of IgSP-hPOMCACTH-IRES-
 5 rTHA-IRES-bDBH-068 fusion
 SEQ ID NO:24 -- DNA sequence oIRES-074
 SEQ ID NO:25 -- DNA sequence of oligo oZeocin-077
 SEQ ID NO:26 -- DNA sequence of oligo oIRES-Zeocin-075
 SEQ ID NO:27 -- DNA sequence of oligo oIRES-Zeocin-076
 10 SEQ ID NO:28 -- DNA sequence IgSP-hPOMCACTH-IRES-rTHA
 -IRES-bDBH-IRES-Zeocin-073
 SEQ ID NO:29 -- DNA sequence of proA+KS
 SEQ ID NO:30 -- DNA sequence of IRES fragment

Deposits

- 15 RINa/ProA/POMC/TH-IRES-DBH cells, transformed
 to produce a catecholamine, an enkephalin and an
 endorphin, as described above in the example (and in
 Table 2), named RINa/ProA/P030/P088, have been
 deposited. The deposit was made in accordance with the
 20 Budapest Treaty and was deposited at the American Type
 Culture Collection, Rockville, Maryland, U.S.A. on June
 7, 1995. The deposit received accession number
 CRL 11921.

- 25 The foregoing description has been for the
 purpose of illustration and description only. This
 description is not intended to limit the invention to
 the precise form exemplified. It is intended that the
 scope of the invention be defined by the claims
 appended hereto.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

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Shou Wong (For purposes of US only)
Joel Saydoff (For purposes of US only)

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(ii) TITLE OF INVENTION: PAIN CELL LINE

(iii) NUMBER OF SEQUENCES: 30

15

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20

(v) COMPUTER READABLE FORM:

25

- (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

30

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

35

(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/481,917
(B) FILING DATE: 07-JUNE-1995

40

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- 56 -

(2) INFORMATION FOR SEQ ID NO:1:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- 10 (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- 15 (iv) ANTI-SENSE: NO
- (vii) IMMEDIATE SOURCE:
(B) CLONE: cONTF-003
- 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCCGGATCG CGTCACCCCT AGAGTCGAGC TGT

33

- 25 (2) INFORMATION FOR SEQ ID NO:2:
- (i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA
- 35 (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

- 40 (vii) IMMEDIATE SOURCE:
(B) CLONE: oIgSP-018

- 45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTTCCCGGGA AAGCGAATT CAC

23

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(2) INFORMATION FOR SEQ ID NO:3:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 849 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- 10 (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- 15 (vii) IMMEDIATE SOURCE:
(B) CLONE: IgSP-hPMC
- 20 (ix) FEATURE:
(A) NAME/KEY: 5'UTR
(B) LOCATION: 1..43
- (ix) FEATURE:
25 (A) NAME/KEY: exon
(B) LOCATION: 44..89
- (ix) FEATURE:
30 (A) NAME/KEY: intron
(B) LOCATION: 90..168
- (ix) FEATURE:
(A) NAME/KEY: 3'UTR
(B) LOCATION: 807..849
- 35 (ix) FEATURE:
(A) NAME/KEY: misc feature
(B) LOCATION: 43..186
(D) OTHER INFORMATION: /product= "IgSp region"
- 40 (ix) FEATURE:
(A) NAME/KEY: misc feature
(B) LOCATION: 187..806
(D) OTHER INFORMATION: /product= "hPMC region"
- 45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

- 58 -

GGATCGGGT CACCCCTAGA GTGAGCTGT GACGGTCTT ACAATGAAT GCAGCTGGT 60
 TATCTCTTC CTGATGGCAG TGGTTACAGG TAAGGGCTC CCAAGTCCA AACTTGAGG 120
 5 TOCATAACT CTGIGACAGT GGCAATCACT TTGCTTTCT TTCTACAGG GTGAATTGG 180
 CTMTCCGGG AAATGGGAC GAGCAGCTC TGACCGAGAA CCCCAGGAG TAGTCATGG 240
 10 GGCATTCCG CTGGGACCA TTGGGCGGC GCATACAGAG CAGCAGGGC AGCAGGGCG 300
 CAGGCAGAA GCGGAGGAC GTCTCAGGG GCGAAGACTG CGGCGCGCTG CCTGAGGGG 360
 GCGCGAGCC CGCAGCGAT GGTGCCAGC CGGCGCGCG CGAGGGCAG CGCTCTACT 420
 15 CCATGGAGCA CTTCGGCTGG GGCAGCGCG TGGCAAGAA GCGCGCGCA GTGAAGGTG 480
 AACCCTAAGG CGCGAGGAC GAGTGGCGG AGGCTTTCC CCTGGAGTTC AAGAGGGAGC 540
 20 TGACTGGCA GCGACTCCG GAGGGAGATG GCGCGAGG CCTGCGCAT GACGGCGAG 600
 GCGCGAGG CGACTGGAG CACAGCTGC TGGTGGCGC CGAGAAGAG GACAGGGCC 660
 OCTACAGGAT GGAGCACTC CGCTGGGCA GCGCGCGCA GGACAAGGC TAGGGGGTT 720
 25 TCATGACTC CGAGAAGAC CAGAGCGCC TGGTACGCT GTTCAAAAC GGCATCATCA 780
 AGAGCGCTA CAGTAGGGC GAGTGGGGC ACAGGGGCG CCAGGGCTAC CCTCCCCAG 840
 30 GAGGTGAC 849

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 35 (A) LENGTH: 525 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- 40 (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- 45 (v) IMMEDIATE SOURCE:

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(B) CLONE: IgSP-hPOMC/DACH

(ix) FEATURE:

(A) NAME/KEY: 5'UTR

5

(B) LOCATION: 1..43

(ix) FEATURE:

(A) NAME/KEY: exon

10

(B) LOCATION: 44..89

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 90..168

15

(ix) FEATURE:

(A) NAME/KEY: exon

(B) LOCATION: 169..482

(ix) FEATURE:

20

(A) NAME/KEY: 3'UTR

(B) LOCATION: 483..525

(ix) FEATURE:

25

(A) NAME/KEY: misc feature

(B) LOCATION: 44..188

(D) OTHER INFORMATION: /product= "IgSP region"

(ix) FEATURE:

30

(A) NAME/KEY: misc feature

(B) LOCATION: 189..482

(D) OTHER INFORMATION: /product= "hPOMC region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

35

GGATCCGGGT CACCCCTAGA GTGAGCTGT GAGGGTCTT ACAATGAAT GCAGCTGGGT 60

TATCTCTTC CIGATGGCAG TGGTACAGG TAAGGGGCTC CCAAGTCCA AACTGAGGG 120

40 TCATAAACT CIGTGACAGT GGCAATCACT TTGCTTTCT TTCTACAGG GTGAATTGG 180

CTTTCCGGC CTTCCTCTG GAGTCAAGA GGGAGCTGAC TGGCAGGGA CTCCGGGAGG 240

GAGATGGGTC CAGAGGCTT GCGATGAGG GCGAGGGGC CCAGGCGAC CTGGAGCACA 300

45

GCTGCTGGT GCGGGGAGG AAGAGGAGG AGGGGCTTA CAGGATGGG CACTTCGGT 360

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GGGGTAGGCC GGGCAGGAC AAGGGCTAGG GGGTTTCAT GAGTCCGAG AAGAGCCAGA 420
 CGGGCTGGT GAGGCTGTC AAAAAGGCA TCATCAAGAA CGCTACAAG AAGGGCGAGT 480
 5 GAGGGCACAG CGGGGGCCAG GGCTAAGCTC CCCCAGGAGG TGGAC 525

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 10 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- 15 (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- 20 (vii) IMMEDIATE SOURCE:
 (B) CLONE: orTH-052
- 25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCCAAGCTTG CACTATGCC ACCCCCAGG 30

30 (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 35 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- 40 (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- 45 (vii) IMMEDIATE SOURCE:
 (B) CLONE: orTH-053

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCCGGATGCT ATGCATTAG CTAATGGCAC

30

5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

20

(vii) IMMEDIATE SOURCE:

(B) CLONE: orTH-054

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCCAAGCTTA TGGTCCCTG GTTCCAAGA

30

30

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- 35 (A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

40

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

45

(vii) IMMEDIATE SOURCE:

(B) CLONE: orTH-078

- 62 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCCAAGCTTC GCCACCATGG TCCCCTGGTT CCC

33

5 (2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

20 (vii) IMMEDIATE SOURCE:

(B) CLONE: oIRES-057

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

25

AAAGGATCG CCCCTCTGCC TCCCCCCCCC

30

(2) INFORMATION FOR SEQ ID NO:10:

30 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

35 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

40 (iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: cbDEH-065

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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AAAGGGGCGG CCCAGGTCA GCGTTGGCC

30

(2) INFORMATION FOR SEQ ID NO:11:

5

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

15

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

20

(B) CLONE: oIRES-bDEH-064

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

25 CTGGCCACAA CCATGTACGG CACGGGGTGG

30

(2) INFORMATION FOR SEQ ID NO:12:

30

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

40

(vii) IMMEDIATE SOURCE:

(B) CLONE: oIRES-bDEH-066

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

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CGCGGTGGG TACATGGTIG TGGCAAGCTT

30

(2) INFORMATION FOR SEQ ID NO:13:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- 10 (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- 15 (iv) ANTI-SENSE: NO
- (vii) IMMEDIATE SOURCE:
(B) CLONE: oIgSP-068
- 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AAAGATATCG CGGCGGGGIC ACCCTAGAG

30

25

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- 30 (ii) MOLECULE TYPE: cDNA
- 35 (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- 40 (vii) IMMEDIATE SOURCE:
(B) CLONE: orTHD-073
- 45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ATACACTGG TCAGAGAGC CGGG

25

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(2) INFORMATION FOR SEQ ID NO:15:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- 10 (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- 15 (vii) IMMEDIATE SOURCE:
(B) CLONE: chPMC-IRES-069
- 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGGGGGAGGG AGAGGGGGOOC GCTGTGGOCT

30

25 (2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1030 base pairs
(B) TYPE: nucleic acid
30 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- 35 (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- 40 (vii) IMMEDIATE SOURCE:
(B) CLONE: rTHD
- (ix) FEATURE:
(A) NAME/KEY: 5'UTR
45 (B) LOCATION: 1..6
- (ix) FEATURE:

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(A) NAME/KEY: exon
(B) LOCATION: 7..1017

(ix) FEATURE:

5 (A) NAME/KEY: 3'UTR
(B) LOCATION: 1018..1030

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

10	AAGCTTATGG T000CTGGTT CCCAAGAAAA GGTGGGAAT TGGACAAGTG TCACCACTG	60
	GTCACCAAGT TTGACCCIGA TCTGGA0CTG GACCA000G GCTTCTCTGA CCAGGIGTAT	120
15	CGCCAG0GTC GGAAGCIGAT TGCAGAGATT G0CTT0CAGT ACAAGCA0GG TGA0CCAATT	180
	CCCATGTGG AATACACAGC GGAAGAGATT GCTA0CTGGA AGGAGGTAATA TGTCAG0CTG	240
	AAGGG0CTCT ATGCTA00CA TGC0T00GG GAGCA0CTGG AGGGTTT0CA GCTTCTGGAA	300
20	CGGTACTGTG GCTA00GAGA GGACAGCATC CCACAGCTGG AGGAG0GTC C0GCTCTTG	360
	AAGGAG0GGA CTGGCTT0CA GCTGGGAC0C GGTGG00GTC TACTGT00GC C0GIGATTTT	420
25	CTGG00CAGTC TGG0CTT00G C0GTGTTTCAA TGCAC0CAGT ATAT00G0CA TGC0T0CTCA	480
	CCATATGCAAT CACCTGAG0C GGACGTGCTC CATGAGCTGT TGGGACATGT A00CATGTTG	540
	GCTGAC0GCA CATTTG00CA GTTCT00CAG GACATTGGAC TTGCATCTCT GGGG00CTCA	600
30	GATGAGAAA TTGAAAACT CT0CAGG0TG TACTGGTTCA CTGTGGAAAT C0GGCTATGT	660
	AATCAGAAATG GGGAGCTGAA GGCTTAATGT GCAGGGCTGC TGCTTT0CTA C0GAGAGCTC	720
35	CTGCACT00C TGTCAGAGGA G0CTGAGGTC C0AG0CTTTG A00CAGACAC AGCAGCTGTG	780
	CAG00CTACC AAGATCAATC CTACCA00CT GTGTACTTTG TGTC0GAGAG CTTCAATGAC	840
	G0CAAGGACA AGCTCAGGAA CTATG00CT C0GTAT0CAG G00CATTCIC TGTCAGTTT	900
40	GAC00GTACA CACTGG0CAT TGAGGTACTG GACAG00CTC ACACCAT0CA G0GCT0CTTG	960
	GAGGGG0T0C AGGATGAGCT GCACAC00CTG G00CAGGCAC TGAGTGG0AT TAGCTAAATG	1020
45	CATAGGAT0C	1030

(2) INFORMATION FOR SEQ ID NO:17:

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(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1037 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
 (B) CLONE: rTHIKS

(ix) FEATURE:
 (A) NAME/KEY: 5'UTR
 (B) LOCATION: 1..13

(ix) FEATURE:
 (A) NAME/KEY: exon
 (B) LOCATION: 14..1024

(ix) FEATURE:
 (A) NAME/KEY: 3'UTR
 (B) LOCATION: 1025..1037

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

	AAGCTTGGGC ACCATGGTCC CCTGGTTCOC AAGAAAAGTG TOGGAAATTGG ACAAGTGTCA	60
35	CCACCTGGTC ACCAAGTTTG AOCCTGATCT GGAOCTGGAC CAOOOGGGCT TCTCTGACCA	120
	GGIGTATGCG CAGGTGGGA AGCTGATTGC AGAGATTGOC TTCCAGTACA AGCAOGGIGA	180
	ACCAATTOOC CATGTGGAT ACACAGOGGA AGAGATTGCT AOCCTGAAGG AGGTATATGT	240
40	CAOBCIGAAG GGCCTCTATG CTACCCATGC CTGCOGGGAG CAOCTGGAGG GTTTCAGCT	300
	TCTGGAACGG TACTGTGGCT AOCGAGAGGA CAGCATOCCA CAGCTGGAGG ACGTGTCCCG	360
45	CTTCTTGAAG GAGOGGACTG GCTTCAGCT GCGAOCOGTG GOCGGTCTAC TGTCCGOCOG	420
	TGATTTTCTG GOCAGTCTGG OCTTCOOGGT GTTTCATGC AOCAGTATA TOCGOCATGC	480

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CTCTCACT ATGCATTAC CTGAGGCGA CTGCTGCAT GAGCTGTGG GACATGTACC 540
 CATGTTGGCT GACGGACAT TTGCGAGTT CTGCGAGAC ATTGGACTTG CATCTCTGG 600
 5 GGCCTCAGAT GAAGAAATG AAAAATCTC CAAGGTGTAC TGGTTCAC TGGAATGG 660
 GCTAATGAAA CAGAATGGG AGCTGAGGC TTATGGTGA GGGCTGCTGT CTTCCTAAG 720
 10 AGAGCTCTG CACTGCTGT CAGAGGAGC TGAGGTGGA GCTTTGACC CAGACACAG 780
 AGCTGTGAG CCTAACAAG ATCAAACTA CAGGCTGTG TACTTTGTG CCGAGAGCT 840
 CAATGAGGC AAGGACAAG TCAGGACTA TGCTCTGTG ATCAGAGGC CATCTCTGT 900
 15 GAAGTTGAC CGTACACAC TGGCATIGA GGTACTGGC AGGCTCACA CCATCAGCG 960
 CTCTTGGAG GGGTCCAGG ATGAGCTGA CAGCTGGC CAGGACTGA GTGCATTAG 1020
 20 CTAAATGCAT AGGATC 1037

(2) INFORMATION FOR SEQ ID NO:18:

- 25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 3425 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- 30 (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- 35 (iv) ANTI-SENSE: NO
- (v) IMMEDIATE SOURCE:
 (B) CLONE: rTH-IRES-bDEH
- 40 (ix) FEATURE:
 (A) NAME/KEY: 5'UTR
 (B) LOCATION: 1..6
- 45 (ix) FEATURE:
 (A) NAME/KEY: exon
 (B) LOCATION: 7..1017

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(ix) FEATURE:
 (A) NAME/KEY: intron
 (B) LOCATION: 1018..1617

5 (ix) FEATURE:
 (A) NAME/KEY: exon
 (B) LOCATION: 1618..3411

10 (ix) FEATURE:
 (A) NAME/KEY: 3'UTR
 (B) LOCATION: 3412..3425

15 (ix) FEATURE:
 (A) NAME/KEY: misc feature
 (B) LOCATION: 1025..1617
 (D) OTHER INFORMATION: /product= "IRES sequence"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

20	AAGCTTATGG TCCCCGGGTT CCCAAGAAAA GGTGCGGAAT TGGACAAGTG TCACCACCTG	60
	GTCACCAAGT TTGACCCGTA TCCTGACCTG GACCACCGG GCTTCTCTGA CCAGGTGTAT	120
25	CGCCAGGGTC GGAAGCTGAT TGCAGAGATT GCTTCCAGT ACAAGCACGG TGAACCAATT	180
	CCCCATGTTG AATACACAGC GGAAGAGATT GCTACCTGGA AGGAGGTATA TGTCAGGCTG	240
	AAGGGCTCTT ATGCTACCA TGGCTGCGG GAGCACTGG AGGGTTTCCA GCTTCTGTAA	300
30	CGGTACTGTG GCTACCGAGA GGACAGCATC CCACAGCTGG AGGAGGTGTC CCGCTTCTTG	360
	AAGGAGGGGA CTGGCTTCCA GCTGGGACC GGGGCGGTC TACTGTGCGC CCGGATTTT	420
35	CTGGCCAGTC TGGCTTCCG CGTGTTTCAA TGCAACCAAT ATATCCGACA TGCTCTCTCA	480
	CCATATGCAAT CACCTGAGCC GGACTGCTGC CATGAGCTGT TGGGACATGT AACCATGTTG	540
	GCCTACCGCA CATTTGCCA GTTCTCCAG GACATTGGAC TTGCATCTCT GGGGCGCTCA	600
40	GATGAGAAA TTGAAAACT CTCACGGTG TACTGGTTCA CTGGGAATT CCGGCTATGT	660
	AAACAGAATG GGGAGCTGAA GGCTTATGGT GCAGGGCTGC TGCTTCTCTA CCGAGAGCTC	720
45	CTGCACTGCC TGTCAGAGGA GCTGAGGTG CAGGCTTTG AACCAGACAC AGCAGCTGTG	780
	CAGGCTACC AAGATCAATC CTACAGGCT GGTACTTTG TGTCAGAG CTTCAATGAC	840

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5 GGCAGGACA AGCTCAGGAA CTATGCTCT OGTATCCAGC GGCATTCTC TGTAAGTTT 900
 GACCGGTACA CACTGGGCAT TGAGTACTG GACAGGCTC ACAOCATCA GCGCTCCTG 960
 10 GAGGGGGTCC AGGATGAGCT GCACAGGCTG GGCAGGCAC TGAGTGCAT TAGCTAATG 1020
 CATAGGATCC GCGCTCTCC CTCCCCCCC OCTAAGTTA CTGGGCGAG CCGCTTGGAA 1080
 15 TTAGGCGGGT GTCGTTTGT CTATATGTA TTTTCCACA TATTGCGTC TTTTGGCAAT 1140
 GTCAGGGCC GGAAGCTGG CCGTCTCTC TTGAGAGCA TTCTAGGGG TCTTTCCCT 1200
 CTCGCCAAG GAATGCAAG TCTGTGAAT GTGTGAAGG AAGCAGTTC TCTGGAGCT 1260
 20 TCTGAAGAC AAACAAGTC TGTAAGGAC CTTTGCAGG AGCGGAACC CCACTGGC 1320
 GACAGGTGC TCTGGGCA AAAGGAGT GTATAAGATA CACTGCATA GCGGCACAA 1380
 25 CCGAGTGC AGTGTGAG TTGGATAGT GTGGAAGAG TCAATGGCT CTCTCAGC 1440
 GTATCAACA AGGGCTGAA GGATGCCAG AAGGTACC ATTGTATGG ATCTATCTG 1500
 GCGCTCGGT GCACATGCT TACATGCTT TAGTGGGT TAAAAAGT CTAGGCCCC 1560
 30 CGAACCAGG GGAAGTGGT TTCTTTGAA AAACAGATG ATAAGCTTC CACAACATG 1620
 TAGGCAAG CCGTGGCGT CTCTCGTC ATCTCGTG CCGACTGCA GCGCTCGCT 1680
 35 CCGCGGAGA GCGCTTCC CTCTCAGC CCGTGGAC CCGAGGGAC CCGTGGCTG 1740
 TCTGGACA TACCTATG GCAGGAGC ATCTACTTC AGCTCTGGT GCGGAGCTC 1800
 AAGCTGGTG TCTGTGTTG GATGTGGC CCGAGGGAG TGGAGAATG TACTTGGTG 1860
 40 GTCCTCTGA CTGACAGGA CCGGCTAC TTTGGGATG CCGTGGTGA CCAGAGGGG 1920
 CAGTCCAC TGGCTCCA GCAGGATC CAGCTCTG GGCACAGG GACTCCAGA 1980
 45 GCGCTGACC TGTCTTCAA GAGGCTTTT GCACTGTG ACGCAACA CTACTCATC 2040
 GAGGAGGCA CCGTCACT GGTGTATGA TTCTGGAG AGCGCTCG GTGCTGGG 2100
 TCCATCAACA CATCGGCTT GCACAGGGG CTGAGAGG TCGAGCTCT GAAGCCAGC 2160
 50 ATCCCAAGC CCGGCTGC CCGGAGAG CCAACATG AGTCCGCG CCGGAGCTC 2220

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	CTCAT0000G G0CAGCAGAC CAGTACTGG TGCTAGTGA C0GAGCT00C GGACGGCTTC	2280
	0000GGCACC ACAT0GTICAT GTACGAG00C AT0GTICAC0G AGGGCA0GA GG0GCTGGTG	2340
5	CA0CACATGG AGGTCCT0CA GTG0G00G0C GAGT0GAGA 0CAT0000CA CTTCAG0GGG	2400
	00CTG0GACT 0CAAGATGA G00GCAG0GG CTCAACTTCT G00GTACGT GCCTG00G0C	2460
	TGG00CTGG G0G0CAAGC CTTTACTAC 0CAGAGGAG CAGG0CTG3C CTTCG3GGG	2520
10	00GGCT0CT 0CAGATTCT 0G0CTGGA GTTCACTAC ACA00CACT GGIGATAACA	2580
	G00GG0G0G ACT0CT0GG CAT0G0CTG TACTAC0GG CTG0GCTG0G G0GCT0GAC	2640
15	G0GGGCATCA TGGAGCTGG C0TGG0GTAC A0G00GTGA TGG0CAT00C 000GCAGGAG	2700
	A0G00CT0G T0CTC00GG CTACTG0AG GACAAGTGA 00CAGCTGGC 0CTG00G0C	2760
	TCAGGGATTC ACATCT0GC CTCTAGCTC CACAGCACC TGAC0G00G GAAGGIGTIC	2820
20	ACAGT0CTGG 0CAGGAC0G 00GGGAGACA GAGAT0GTA ACAGGGACAA 0CACTACAGC	2880
	0CACACT0C AGGAGAT0G CATGTTGAAG AAGT0GTGT CTGT0CAG0C GGGAG0GTG	2940
25	CTCATCA0CT CTTCACATA CACA0GGA GACAGGAGC TGG0CA0GT GGGGGCTTC	3000
	GGGAT0CTGG AGGAGATGTG 0GTCACTAT GTGCACTACT A0000CAGC GCAGCTGGAG	3060
	CTCTGCAAGA G0G0GTGGA 00CTGGCTTC CTGCACAAGT ACTT0G0CT 0GTGAACAGG	3120
30	TTCAACAG0G AGGAAGTCTG CACCTG000C CAGG0GTCTG T00CTAGCA GTTGT0CTC	3180
	GTG00CTGA ACT0CTTCAA 00G0GAGGTG CTCAAG00C TGTA0GGCTT 0GCA00CATC	3240
35	T0CATGCACT GCAACAGGT C0G0G0GT C0CTT0CAGG G0GAGTGGAA T0GGCAG00C	3300
	CTG0CTGAGA T0GTGT0CAG GTTGAAGAG 00CA00CTC ACTG00CAGC CAG0CAGCT	3360
	CAGAG000G 00G0000AC 0GTGCTGAAC ATCAGTGGG GCAAGGCTG AACGTGG0G	3420
40	G00GC	3425

(2) INFORMATION FOR SEQ ID NO:19:

- 45 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 3432 base pairs
 (B) TYPE: nucleic acid

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(C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

5 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

10 (vii) IMMEDIATE SOURCE:
 (B) CLONE: rTHIKS-IRES-bDEH

(ix) FEATURE:
 15 (A) NAME/KEY: 5'UTR
 (B) LOCATION: 1..13

(ix) FEATURE:
 20 (A) NAME/KEY: exon
 (B) LOCATION: 14..1024

(ix) FEATURE:
 25 (A) NAME/KEY: intron
 (B) LOCATION: 1025..1624

(ix) FEATURE:
 (A) NAME/KEY: exon
 (B) LOCATION: 1625..3418

30 (ix) FEATURE:
 (A) NAME/KEY: 3'UTR
 (B) LOCATION: 3419..3432

(ix) FEATURE:
 35 (A) NAME/KEY: misc_feature
 (B) LOCATION: 1032..1624
 (D) OTHER INFORMATION: /product= "IRES sequence"

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AAGCTTGGOC ACCATGGTCC CCTGGTTCOC AAGAAAAGTG TOGGAATTGG ACAAGTGTCA	60
CCACCTGGTC ACCAAGTTTG ACCCGATCTT GGACCTGGAC CACCCGGGCT TCTCTGACCA	120
GGIGTATOGC CAGGTGGGA AGCTGATTGC AGAGATTGOC TTCCAGTACA AGCAGGGTGA	180

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	ACCAATTUCC CATGIGGAAT ACACAGOGGA AGAGATTGCT AOCIGGAAGG AGGTATATGT	240
	CACGCTGAAG GGOCTCTATG CTACCCATGC CTGCGGGGAG CAOCIGGAGG GTTTCAGCT	300
5	TCIGGAAGG TACTGTGGCT AOCGAGGGA CAGCATUCCA CAGCTGGAGG AOGIGUCCG	360
	CTTCITGAAG GAGGGACTG GCTTCAGCT GCGAOCOGTG GCGGTCTAC TGTCGCGCG	420
	TGATTTCTG GOCAGTCTGG OCTTCGGGT GTTTCATGC AOCAGTATA TOCGCATGC	480
10	CTCCTCACT ATGCATTAC CTGAGCGGA CTGCTGCCAT GAGCTGTGG GACATGTACC	540
	CATGTGGCT GAOGCACAT TTGCGAGTT CTCGAGGAC ATGGACTTG CATCTCGG	600
15	GGOCTCAGAT GAAGAATTG AAAACTCTC CACGGGTAC TGGTTCATG TGAATTGG	660
	GCTATGTAAA CAGAATGGG AGCTGAAGC TTATGGTGA GGGCTGCTGT CTTCCTAGG	720
	AGAGCTCTG CACTOCTGT CAGAGGAGC TGAGGTGGA GCTTTGAC CAGACAGC	780
20	AGCTGTGAG CCTACCAAG ATCAAACCTA CCAGCTGTG TACTTTGTG CCGAGCTT	840
	CAATGAGGC AAGGACAAG TCAGGAATA TGCTCTGT ATCCAGGOC CATCTCTGT	900
25	GAAGTTTGA CCGTACACAC TGGCATTA CTTACTGGAC AGOCTCACA CCATCCAGG	960
	CTCCTGGAG GGGTCCAGG ATGAGCTGA CACCTGGCC CAGCACTGA GTGCCATTAG	1020
	CTAAATGCAT AGGATCGGC OCTCTOCTC CCCCCOCT AAGTTACTG GCGAAGCG	1080
30	CTTGAATAA GGOGGGTG CGTTGTCTA TATGTTATT TCACCATAT TGOGTCTT	1140
	TGCAATGTG AGGGCGGGA AOCIGGOC TGCTTCTTG ACGAGCTT CTAGGGCT	1200
35	TTCCCCCTC GCAAGGGA TGCAAGGTCT GTTGAATGT GTGAGGAAG CAGTCTCT	1260
	GGAAGCTCT TGAAGACAA CAGGTCTGT AGGACCTT TGAGGCAGC GGAACCCCC	1320
	AOCIGGAGC AGGTGCTCT GGGGCAAAA GCAAGGTGA TAAGATAC CTTCAAGGC	1380
40	GCACACCC CAGTCCAGG TTGTGAGTG GATAGTTTG GAAAGGTCA AATGGCTCT	1440
	CTCAAGGTA TTCAACAGG GGTGAAGG TGCCAGAG GTACCCATT GTATGGGATC	1500
45	TGATCTGGG OCTGGGTGA CATGCTTAC ATGTGTTAG TCAGGTAA AAAAGCTCA	1560
	GGCCCCCGA AOCAGGGA CGTGGTTTC CTTGAAAA CAGATGATA AGCTTGCCAC	1620

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AACCATGTAC GGCACGCGG TGGCGGCTT CCTGGTCATC CTGGTGGCTG CACTGCAGGG 1680
 CTGGCTTCCC GCGGAGAGC CCTTCCCCTT CCACATCCCC CTGGACCCCG AGGGGACCT 1740
 5 GGAGCTGTCC TGGACATCA GCTATGGCA GGAGACATC TACTTCCAGC TCGTGGTGG 1800
 GGAGCTCAG GCTGGGTCC TGTTTGGAT GTGGACCA GGGGAGCTGG AGATGCTGA 1860
 10 CTGGTGGTG CTCTGGACTG ACAGGACGG CGCTACTTT GGGATGCTT GGATGACCA 1920
 GAAGGGGAG GTCCACTGG ACTCCAGCA GGATTACAG CTCTGGGGG CACAGAGGC 1980
 TCCAGAGGC CTGTACTGC TCTCAGAG GCTTTTGGC AACTGAGAC CCAAGACTA 2040
 15 CTTATCGAG GACGGCACG TCCACTGGT GTATGGATC CTGGAGGAGC CGTCCGGTC 2100
 GCTGGAGTCC ATCAACATC CCGCTTCCA CAGGGGCTG CAGAGGGTCC AGCTGCTGA 2160
 20 GGCAGCATC CCAAGCGG CCGTGGCGC GGCACGGC ACCATGGAGA TCCGCGCCC 2220
 CGAGTCTTC ATCCCGGC AGCAGACAC GTACTGGTC TAGTGACG AGCTCCCGA 2280
 CGGCTTCCC CGCACACA TGTATGTA CGAGCCATC GTACCGAGG CCAAGAGGC 2340
 25 GCTGGTCC CACATGGAG TCTTCCAGT CGCGCGGAG TTGAGACCA TCCCCACTT 2400
 CAGCGGCCC TGGACTCCA AGATGAGC GCAGGGCTC AACTTCTGC GTACGTGCT 2460
 30 GCGCGCTGG GCGTGGGG CCAAGGCTT TTACTACCA GAGGAGCAG GCTGGGCTT 2520
 CGGGGGCCC GGTCTTCCA GATTCTCG CCTGGAGIT CACTACACA ACGACTGGT 2580
 GATACAGGC CGCGCGACT CCGGGCAT CCGCTGTAC TACAGGCTG CGCTGGGG 2640
 35 CTGACGGG GGCATCATG AGCTGGGCT GCGTACAG CCGTGATGG CCATCCCCC 2700
 GTAGGAGAG GCTTGTTC TCAAGGCTA CTGACGGC AAGTGACCC AGCTGGGCT 2760
 40 GCGCGCTCA GGTATCACA TCTTGGCTC TCGCTCCAC AGCACTGA CCGCGCGA 2820
 GGTGGTACA GTCTGGCA GGGAGCGG GGCACAGAG ATGTGACA GGCACACA 2880
 CTACAGCCA CACTCCAG AGATCGCAT GTTGAGAG GTGTGTCTG TCCAGCGG 2940
 45 AGAGTCTC ATCACTCTT GCACATACA CAGGAGAC AGGAGGCTG CCAAGTGG 3000

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GGGCTTGGG ATCTGGAGG AGATGTGGT CACTATGTG CACTACTACC CCCAGACCA 3060
 GCTGGAGCTC TGCAAGAGG CCGTGGACC TGGCTTCTG CACAAGTACT TCGGCTGGT 3120
 5 GAACAGGTC AACAGGAGG AAGTCTGAC CTGCCCCAG GGTCTGTTC CTGAGTGT 3180
 TGGCTGGG CCGTGGACT CCTTCAAG CGAGGTCTC AAGGCTGT AGGCTTGC 3240
 ACCATCTTC ATGCTGCA ACAGTCTC GGGGTGGC TTCCAGGGG AGTGAATG 3300
 10 GCAGGGCTG CCGTGGATG TGTCCAGGT GGAAGAGCC ACCCTTACT GGCAGGAG 3360
 CCAGGCTAG AGCCCCCG GGGCAGGT GCTGAACATC AGTGGGGCA AAGCTGAC 3420
 15 GGGGGGGC GC 3432

(2) INFORMATION FOR SEQ ID NO:20:

- 20 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 25 (ii) MOLECULE TYPE: cDNA
 (iii) HYPOTHETICAL: NO
 (iv) ANTI-SENSE: NO
 30 (vii) IMMEDIATE SOURCE:
 (B) CLONE: chPMC-IRES-070

- 35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AGGGCACAGC GGGGGCTCT CCTTCCCCC 30

- 40 (2) INFORMATION FOR SEQ ID NO:21:

- 45 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

5 (iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: oIRES-rTHD-071

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GAAOCAGGEG ACCATGGTIG TGGCAAGCTT

.30

15

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

20

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

25

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

30

(vii) IMMEDIATE SOURCE:

(B) CLONE: oIRES-rTHD-072

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CTTGCCACAA CCATGGTCC CTGGTTCCCA

30

(2) INFORMATION FOR SEQ ID NO:23:

40

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4499 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

45

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- 5
- (vii) IMMEDIATE SOURCE:
(B) CLONE: pmtc-th-dbh fusion
- 10 (ix) FEATURE:
(A) NAME/KEY: 5'UTR
(B) LOCATION: 1..43
- (ix) FEATURE:
15 (A) NAME/KEY: exon
(B) LOCATION: 44..89
- (ix) FEATURE:
20 (A) NAME/KEY: intron
(B) LOCATION: 90..168
- (ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 169..482
- 25 (ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 483..1080
- 30 (ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1081..2091
- (ix) FEATURE:
35 (A) NAME/KEY: intron
(B) LOCATION: 2092..2691
- (ix) FEATURE:
40 (A) NAME/KEY: exon
(B) LOCATION: 2692..4485
- (ix) FEATURE:
45 (A) NAME/KEY: 3'UTR
(B) LOCATION: 4486..4499
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

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GGGGCGGGT CACCCCTAGA GTGAGCTGT GAGGTCTTT ACAATGAAT GCAGCTGGT 60
 TATCTCTTC CTGATGGCAG TGGTACAGG TAAGGGGCTC CCAAGTCCA AACTTGAGG 120
 5 TOCATAACT CTGTCAGT GGCAATCACT TTGCTTTCT TTCTACAGG GTGAATTGG 180
 CTTTCCGGC CTTCCCTG GAGTCAAGA GGGAGCTGAC TGGCAGCA CTTCCGGAG 240
 10 GAGATGGCC CGAGGCTT GCGATGAG GGCAGGGC CAGGCTGC CTGGAGACA 300
 GCTGCTGGT GGGGCGAG AAGAGGAG AGGGGCTCA CAGATGGG CACTTGGCT 360
 GGGCAGCC GCGAAGGAC AAGGCTAG GGGTTTAT GACTCGAG AAGAGCAGA 420
 15 GGGGCTGGT GAGCTGTTC AAAACGCA TCATCAGAA GCGTACAG AAGGGGAGT 480
 GAGGCACAG GGGGCGCTC TCCCTCCCT CCGCTACG TTAGTGGG AAGGGCTTG 540
 20 GAATAAGCC GGTGTGGT TGCTATATG TTATTTCCA CCAATGCT GCTTTTGGC 600
 AATGAGGG CCGGAAC TGGGCTGTC TTCTGAGA GATTCCTAG GGTCTTTC 660
 CCTCTGCA AAGGAATGA AGGTCTGTG AATGTGTGA AGGAGCAGT TCTCTGGAA 720
 25 GCTCTTGA GACAAAC GCTGTAGG ACGTTTGA GGCAGGGA CCGGCACT 780
 GCGACAGG GCTCTGGG CCAAGGCA CGTGTATAG ATACCTGC AAGGGGCA 840
 30 CACCCAGT GCGAGTGT GAGTGGATA GTTGGGAA GAGTCAATG GCTCTCTCA 900
 AGGTATTCA ACAGGGCT GAGGATGC CAGAGGTAC CCAATTGT GGTCTGAT 960
 CTGGGCTC GGTGACATG CTTTACATG GTTAGTGA GGTAAAAA GGTCTAGCC 1020
 35 CCGGACCA GGGGAGTG GTTTCTTT GAAACACG ATGATAGCT TGGACACC 1080
 ATGGTCCCT GGTCCCAAG AAAAGTGTG GATTTGCA AGTGTACA CTTGGTACC 1140
 40 AAGTTGAC CTGATCTGA CTTGACAC CCGGCTTCT CTGACAGT GTATGCGAG 1200
 GGTGGAGC TGATTCAGA GATGCTTC CAGTACAG AGGTGAAC AATTCCCAT 1260
 GTGATACA CAGGAGA GATGCTAC TGAAGGAG TATATGTC GCTGAAGGC 1320
 45 CTCTATCTA CCAATGCT CCGGAGAC CTGGAGGT TOCAGCTCT GGAAGGTAC 1380

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	TGTGGCTAOC GAGAGGACAG CATUCCACAG CTGGAGGAG TGTCUUGCTT CTGAAGGAG	1440
	CGGACTGGCT TOCAGCTGG ACOUGTGGOC GGTCTACTGT CCGUUGGIGA TTTTCTGGOC	1500
5	AGTCTGGCT TOCGGTGTT TCAATGCACC CAGTATATOC GGCATGGCTC CTCACCTATG	1560
	CATTCACTG AGCGGACTG CTGCCATGAG CTGTGGGAC ATGTACCAT GTTGGCTGAC	1620
	CGCACATTG CCGAGTCTC CCAGGACATT GGACTTGCAT CTCGGGGGC CTCAGATGA	1680
10	GAAATTGAA AACTCTCCAC GGIGTACTGG TTAATGTGG AATGGGGCT ATGTAAACAG	1740
	AATGGGGAGC TGAAGCTTA TGGTGCAGG CTGCTGTCTT CTTAGGAGA GCTCTGCAC	1800
15	TUOCTGTAG AGGAGCTGA GGTUUGAGC TTTGACUAG ACACAGGAGC TGTCAGUOC	1860
	TACCAAGATC AAACCTACA GCTGTGTAC TTTGTGTUOG AGAGCTTCAA TGACGOCAG	1920
	GACAAGCTCA GGAATATGC CTCGTGTATC CAGCGUOCAT TCTCTGTGA GTTGAUUG	1980
20	TACACACTGG CCATTGAGT ACTGGACAGC CTTACACCA TCCAGGCTC CTGGAGGGG	2040
	GTCAGGATG AGCTGCACAC CTTGGUOCAC GCACTGAGTG CCATTAGCTA AATGCATAGG	2100
25	ATUUGUUUCT CTTCTUOC CUCUCTAAC GTTACTGGC GAAGUUGCTT GGAATAGGC	2160
	CGGTGTGGT TTGTCTATAT GTTATTTTC ACCATATGC CGTCTTTTG CAATGTGAGG	2220
	GUUGGAAC CTGGUUGCT CTCTGTAGC AGCATTCCTA GGGGTCTTC CUCCTUGOC	2280
30	AAAGGAATGC AAGGTCTGT GATGTGTGT AAGGAAGCAG TTCTCTGGA AGCTCTTGA	2340
	AGACAAPCAA CGTCTGTAC GACCTTTGC AGGCAGGGA AUCUUCACC TGGGACAGG	2400
35	TGCTCTGGG GCAAAAGCC AGGTGTATA GATACUCTG CAAAGGGGC ACAACUOCAG	2460
	TGCCAGTGT TGAGTGGAT AGTGTGGAA AGATCAAT GGTCTCTC AAGGTATTC	2520
	ACAAGGGGC TGAAGATGC CCAGAGGTA CUCATTGTA TGGATCTGA TCTGGGCT	2580
40	CGGTGCACAT GCTTACATG TGTGTAGTG AGGTAAAAA AGTCTAGGC CUCUGAAC	2640
	ACGGGAAGT GGTTTTCTT TGAAPACAC GATGATAAGC TTGCCACAAC CATGTAGGC	2700
45	ACCGGGTGG CCGTCTCTT GTGATCTC GTGGTGCAC TGCAGGGCTC GGTUUGGC	2760
	GAGAGUUCCT TUCCTTCA CATUUCCTG GACUUGAGG GGAUUCGGA GCTGTCTG	2820

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	AACATCAGCT ATGCGCAGGA GACCATCTAC TTCCAGCTCC TGGTGGGGGA GCTCAAGGCT	2880
	GGTGTCTGT TTGGGATGTC GGACCGAGGG GAGCTGGAGA ATGCTGACTT GGTTGGTCTC	2940
5	TGGACTGACA GGGACGGGCG CTACTTTGGG GATGCGTGGG GTGACCAGAA GGGGCAGGTC	3000
	CACTGGACT CCCAGCAGGA TTACACGCTT CTGGGGGCAC AGAGGACTCC AGAAGGCGTC	3060
10	TACCTGCTCT TCACAGGGCC TTTTGGGCAC TGTGACCCCA AGGACTACCT CATCGAGGAC	3120
	GGCAACGGTCC AACTGGTGTA TGGATTCTTG GAGGAGCGGC TCGGTGGCTT GGAGTCCATC	3180
	AACACATCCG GCTTGCACAC GGGGCTGCAG AGGGTGCAGC TCGTGAAGCC CAGCATCCCC	3240
15	AAGCGCGGCC TGGCGGGGA CAGCGGCACC ATGGAGATCC GCGCGCGGA CGTCTCATC	3300
	CGCGGCAGC AGACCAAGTA CTGGTCTTAC GTGACCGAGC TCGCGACGG CTTCGCGGG	3360
20	CACCATATCG TCATGTACGA GCGCATGGTC ACGAGGGGCA AGGAGGCGCT GGTGCACCAC	3420
	ATGGAGGCTT TCCAGTGGC CGCGAGTTC GAGACCATCC CCACTTCCAG CGGGCGCTGC	3480
	GACTCCAGA TGAAGCGCA GCGGCTCAAC TTCTGCGTTC AGTGTCTGGC CGCTTGGGCC	3540
25	CTGGCGGCA AGGCTTTTA CTACCCAGAG GAAGCAGGCC TGGCTTGGG GGGCGCGGC	3600
	TCCTCCAGAT TTCTCGGCT GGAAGTTCAC TAACCAACC CACTGGTGAT AACAGCGCGG	3660
30	CGGACTCTT CGGGCATCCG CCGTACTTAC ACGGCTGGC TGGCGGCTT CGACGCGGC	3720
	ATCATGGAGC TGGGCTGGC GTACACGCGC GTGATGGCA TCGCGCGCA GGAGACGGC	3780
	TTGTCTTCA CGGCTACTG CAGGACAAG TGCACCCAGC TGGGCTGGC CGCTTACGG	3840
35	ATTACATCT TCGCTCTCA GCTCCACAG CACTGACCG GCGGAGGT GTTCACAGT	3900
	CTGGCCAGG ACGCGCGGA GACAGAGATC GTGAACAGG ACAACACTA CAGCCACAC	3960
40	TTCCAGGAGA TCGCATGTT GAAGAAGTTC GTGTCTGTC AGCGGGAGA CGTCTCATC	4020
	ACCTCTTGA CATACACAC GGAAGACAG AGGCTGGCA CGTGGGGG CTTCGGGATC	4080
	CTGGAGGAGA TGTGGTCAA CTATGTGCAC TACTACCCC AGACGAGCT GGAGCTCTG	4140
45	AAGAGCGCG TGGACCTGG CTCTCTGCAC AAGTACTTCC GCTGTGTGA CAGGTTCAC	4200

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AGGAGGAG TCTGCACTG CCCCAGGCG TCTGTCCCTG AGCAGTTTGC CTCGGTGGC 4260
TGGAACTCCT TCAACCGGA GGIGCTCAAG GGCCTGTAGG GCTTGGCACC CATCTCCATG 4320
5 CACTGCAPCA GGTCCTGGC OGTCGGCTTC CAGGGGAGT GGAATUGGCA GGCCTGCT 4380
GAGATGGTGT CCAGGTGGA AGAGCCACC CCTCCTGTC CAGCCAGCA GGCCTAGAGC 4440
CCCGGGGGC CCAAGGTCT GACATCAGT GGGGGCAAG GCTGAACTG GGGGGGGC 4499

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(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
15 (B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

25

(vii) IMMEDIATE SOURCE:

(B) CLONE: oIRES-074

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

AAAGGGGGG CCGCTCTCC TCCCCCCC

30

(2) INFORMATION FOR SEQ ID NO:25:

35

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
40 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

45

(iv) ANTI-SENSE: NO

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- (vii) IMMEDIATE SOURCE:
(B) CLONE: oZeocin-077

5

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

AAACTGGAGT CAGTCTGCT CCTGGGCAC

30

- 10 (2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
15 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- 20 (iii) HYPOTHETICAL: NO

- (iv) ANTI-SENSE: NO

- 25 (vii) IMMEDIATE SOURCE:
(B) CLONE: OIRES-Zeocin-075

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

30

GGTCACTTG GCGATGGTTG TGGCAAGCTT

30

- (2) INFORMATION FOR SEQ ID NO:27:

- 35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

40

- (ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO

- 45 (iv) ANTI-SENSE: NO

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(vii) IMMEDIATE SOURCE:

(B) CLONE: oIRES-Zeocin-076

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CITGGCACAA CCATGGGCGAA GTTGACCACT

30

(2) INFORMATION FOR SEQ ID NO:28:

10

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5540 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

15

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

20

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

25

(B) CLONE: POMCDACTH-IRES-THD-IRES-DBH-IRES-Zeocin

(ix) FEATURE:

(A) NAME/KEY: 5'UTR

(B) LOCATION: 1..118

30

(ix) FEATURE:

(A) NAME/KEY: exon

(B) LOCATION: 119..164

35

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 165..243

40

(ix) FEATURE:

(A) NAME/KEY: exon

(B) LOCATION: 244..557

45

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 558..1155

(ix) FEATURE:

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(A) NAME/KEY: exon
(B) LOCATION: 1156..2166

5 (ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 2167..2766

10 (ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 2767..4560

15 (ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 4561..5159

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 5160..5534

20 (ix) FEATURE:
(A) NAME/KEY: 3'UTR
(B) LOCATION: 5535..5540

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

AAGCTTGGTA	COGAGCTGG	ATOCCTAGT	AAOGBGOC	AGTGTGCTGG	AATTCIGCAG	60
ATATOCATCA	CACIGGGGC	CGGTCACOC	CTAGAGTGA	GCTGTGAGG	TOCTTACAAT	120
30 GAAATGCAGC	TGGGTATCT	TCTTCTGAT	GGCAGTGGT	ACAGGTAGG	GGCTOCCAAG	180
TOCCAACCTT	GAGGTTCAT	AACTCTGIG	ACAGTGCAC	TCACTTTGOC	TTTCTTTCTA	240
35 CAGGGGIGAA	TTGGCTTTC	CGGCTTTC	COCTGGAGT	CAAGAGGGAG	CTGACTGGCC	300
AGGACTTGG	GGAGGAGAT	GGGCGGAG	GGCTGGGA	TGAGGGGCA	GGGGGAGG	360
COGACTGGA	GCACAGCTG	CTGGTGGGG	COGAGAGAA	GGAGAGGGC	COCTACAGGA	420
40 TGGAGTACTT	CGCTGGGGC	AGGCGGCA	AGGACAAGG	CTAGGGGGT	TTATGACT	480
COGAGAGAG	CCAGAGGOC	CTGGTGAOC	TGTTCAAAA	CGCATCATC	AAGAGGCT	540
45 ACAAGAGGG	CGAGTGGG	CACAGGGGC	COCTCTOCT	CCCCCCCCC	TAACTTACT	600
GGCGAGGC	GCTTGGATA	AGGCGGGGT	GGTTTGTCT	ATATGTTATT	TTCAACATA	660

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	TTGCGGCTTT TTGGCAATGT GAGGGGCGG AAACCTGGCC CTGCTTCTTT GAGAGCATTT	720
	OCTAGGGGTC TTTCGCTCTT CGCCAAAGGA ATGCAAGGTC TGTGAATGT CGTGAAGGAA	780
5	GCAGTTCCTC TGGTACCTTC TTGAGACAA ACAAGCTCTG TAGGACCTT TTGCAGGCAG	840
	CGGACGCTTC CACCTGGCGA CAGGTGCTTC TGCGGCGAAA AGCAGCTGT ATAAGATACA	900
10	OCTGCAAGG CGGCACAAC CCAGTGCAC GTTGTGAGTT GGATAGTTGT GGAAGAGTC	960
	AAATGGCTCT OCTCAGGT ATTCAACAAG GGGCTGAGG ATGCCAGAA GGTAACCAT	1020
	TGTATGGGAT CTGATCTGGG GCTCGGTGC ACATGCTTTA CATGCTTTA GTGAGGTTA	1080
15	AAAAAGCTT AGGGGCGCG AACACGGGG ACGTGGTTTT CTTTGAAAA ACAGATGAT	1140
	AAGCTTGC CAACATGGT CCGCTGGTC CCAAGAAAAG TGTGGGATT GGACAGTGT	1200
20	CACCACTGG TCACCAAGTT TGACCTGAT CTGCACTGG AACACGCGG CTCTCTGAC	1260
	CAGGTGATC GCGAGGTGC GAGCTGATT GCAGAGTTG CTTCCAGTA CAGCAAGGT	1320
	GACCAATTC CCAATGTTG ATACACAGG GAGAGATTG CTACCTGGAA GGAGGTATAT	1380
25	GTACGCTGA AGGGCTCTA TGTACCAT GCTCGCGG AGCACTGGA GGGTTTCAG	1440
	CTCTGGTAC GGTACTGGG CTACCGAGG GACAGCATC CACAGCTGA GGAGGTGTC	1500
30	CGCTCTTGA AGGAGCGAC TGGCTTCAG CTGCGACCG TGGCGGCTT ACTGTGCGC	1560
	CGGATTTTC TGGCAGCTT GGGCTTCGC GTGTTTCAAT GCACCCAGTA TATCGGCAT	1620
	GCTCTCTAC CTATGCTTC AACTGAGCG GACTGCTGC ATGAGCTGT GGCATGTA	1680
35	CCATGTTGG CTGACCGAC ATTGCGCG TTTCCAGG ACATGGACT TGCATCTCTG	1740
	GGGCTCTAG ATGAGGAAT TGAAAACTC TCACGGTGT ACTGGTTCAC TGTTGAATC	1800
40	GGCTATGTA AACAGATGG GGAGCTGAG GCTTATGGG CAGGCTGCT GTCTCTTAC	1860
	GGAGAGCTC TGCCTGCT GTGAGGAG OCTGAGTTC GAGCTTTGA CCGACACA	1920
	GCAGCTGTC AGGCTTACA AGATCAACC TACAGCTG TGTACTTTGT GTGAGAGC	1980
45	TTCATGAGC CCAAGGACA GCTCAGTAC TATGCTCTC GTATCCAGG CCAATCTCT	2040

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	GTCAGATTG ACCGTACAC ACTGGCCATT GACGTACTGG ACAGCCCTCA CACCATCCAG	2100
	CGCTCCTTGG AGGGGGTCCA GGATGAGCTG CACACCTTGG CCCAGGCACT GAGTGCCATT	2160
5	AGCTAAATGC ATAGGATCCG CCCCCTCCG TCCCCCCCC CTAAGGTAC TGGCGAAGC	2220
	CGCTTGGAAAT AAGGCGGGTG TGGTTTGTG TATAATTTAT TTCCACCAT ATTGCGTCT	2280
	TTTGGCAATG TGAGGGGCGG GAACCTGGC CCTGCTCTCT TGACGAGCAT TCCTAGGGGT	2340
10	CTTTCCCCC TOGCCAAGG AATGCAAGG CTGTGTAATG TGTGAAGGA AGCAGTTCT	2400
	CTGGAGCTT CTGAAGACA AACAGGCT GTAGGAGCC TTTCAGGCA GCGGAACCC	2460
15	CCACCTGGG ACAGGTGCT CTGGGCGAA AAGCCAGTG TATAAGATC ACCTGCAAG	2520
	GCGGCACAC CCCAGTGCA CGTTGTGAGT TGGATAGTTG TGGAAAGAT CAAATGGCTC	2580
	TCCTCAGCG TATTCACAA GGGGCTGAG GATGCGGAGA AGGTACCCCA TTGTATGGA	2640
20	TCGTATCTGG GGCCTGGTG CACATGCTTT ACATGTGTTT AGTCAGGTT AAAAAAGTC	2700
	TAGCCCCCC GAACACGGG GACGTGGTTT TCCTTTGAA AACACGATG TAAGCTTGGC	2760
25	ACAAACATGT ACGGCACGC GGTTGGGTC TTCTGGTCA TCCTGGTGG TGCATGCG	2820
	GGCTGGCTC CCGCGAGAG CCCCCTCCG TTCCACATC CCGTGGACC CGAGGGGAC	2880
	CTGGAGCTGT CCTGACAT CAGCTATGG CAGGAGACA TCTACTTCA GCTCCTGGTG	2940
30	CGGGAGCTCA AGGCTGGTGT CCCTTTTGG ATGTGGGAC GAGGGAGCT GGAGAATGCT	3000
	GACTTGGTGG TGCTCTGGC TGACAGGGC GGGGCTACT TTGGGGATG CTGGAGTGAC	3060
35	CAGAGGGGC AGGTCCACT GGTCTCCAG CAGGATACC AGCTCTGGG GGCACAGAG	3120
	ACTCCAGAG GCTGTACT GCTCTTCAAG AGGCTTTTG GCACTGTGA CCCCACGAC	3180
	TACCTCATG AGGACGGCAG CGTCCACTG GTGTATGGAT TCCTGGAGGA GCGCTCGG	3240
40	TGCTTGGAGT CCATCACAC ATCGGCTTG CACAGGGGC TGACAGGGT GCAGCTGCTG	3300
	AAGCCAGCA TCCCCAGC GGGCTGGC GGGACAGC GCACATGGA GATCGGCGC	3360
45	CCGAGGTC TCATCCCCG CAGCAGAC AGGTACTGGT GCTAGTGAC CGAGCTCCG	3420
	GACGGCTTC CCGGCACCA CATGTGATG TAGAGGCA TGTACCGA GGCACAGAG	3480

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GCGCTGGTGC ACCACATGGA GGTCTTCCAG TGCGCGCGCG AGTTCGAGAC CATCCCCAC 3540
 TTCAGCGGGC OCTGGGACTC CAGATGAAG CGCGAGCGGC TCACTTCTG CCGTCAAGTG 3600
 5 CTGGCGCGCT GGGCGCGGG CGCGAGGCG TTTTACTACC CAGAGGAAG AGGCGTGGC 3660
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 10 GTGATACAG GCGCGCGGA CTCTCGGC ATCGCGCTGT ACTACAGGC TCGCTGGG 3780
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 15 CTGCGCGCT CAGGATTCAT CTCTTGGC TCTAGCTCC ACAGCGACT GACCGCGGG 3960
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 20 CACTACAGC CACTTCCA GAGATCGC ATGTGAGA AGGTGTGTG TGTCCAGCG 4080
 GGAGAGTGC TCATCCTC TTGCATAC AACCGGAG ACAGGAGCT GCGACCGTG 4140
 GGGGCTTGG GATCTGGA GAGATGTG GTCACTATG TGCCTACTA CCGCAGAG 4200
 25 CAGCTGGAG TCTGAGAG CGCGTGGC CTGCTTCC TGCACAGTA CTCTCGCTC 4260
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 30 TTTGCTCGG TGCGTGGAA CTCTTCAAC CGGAGGTG TCAGCGCT GTAGGCTT 4380
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 45 CTCTTGAAG ACAACAGG TCTGTAGA CCGTTGAG GAGCGGAC CCGCACTG 4860

- 88 -

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 15 TGGTGGGA CCAAGTGC CTGTATCA GGGGGTCA GCAAGGIG GTGGGACA 5340
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 TGTGTGCAC GACTTGG GAGGCTGG GGGGGCAT GAGGAGTC GGGAGGAGC 5460
 20 CGTGGGGGG GGAGTGGC CTGGGGAC GGGGGGCA CTGGTGCAC TTGTGGGG 5520
 AGGAGGAG CACTGG 5540

25 (2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 829 base pairs
 (B) TYPE: nucleic acid
 30 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

35 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

40 (vii) IMMEDIATE SOURCE:

(B) CLONE: ProAKS

(ix) FEATURE:

- (A) NAME/KEY: 5'UTR
 45 (B) LOCATION: 1..16

(ix) FEATURE:

- 89 -

(A) NAME/KEY: exon
(B) LOCATION: 17..820

(ix) FEATURE:

5 (A) NAME/KEY: 3'UTR
(B) LOCATION: 821..829

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

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15	CGGCTAGTGC CGCGGGGCGG ACATCAACTT CCTGGCTTGC GTAAATGGAAT GCGAAGGTAA	180
	ACTGCTTCTT CTGAAATTTT GGGAACTG CAAGGAGCTC CTGCAGCTGT CCAAPCCAGA	240
	GCTTCTCTAA GATGGCAACA GCAOCTCAG AGAAATAGC AAACGGGAG AAAGCATTT	300
20	GCTAGCCAAA ACGTATGGGG GCTTCATGAA AAGGTATGGA GGCTTCATGA AGAAATGGA	360
	TGAGCTTTAT CCGATGGAGC CAGAAGAGA GGCAATGGA AGTGAATCC TCGCCAAGG	420
25	GTATGGGGGC TTCAATGAGA AGGATGCAGA GGAGGAGAC TCGCTGGCCA ATTCTCAGA	480
	CTGCTAATAA GAGCTTCGGG AAACAGGGGA CACCGAGAG CGTAGCCACC ACCAGGATGG	540
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30	AAGCCCCCAA CTGGAAGATG AAGCCAAAGA GCTGCAGAAG CGATATGGGG GCTTCATGAG	660
	AAGAGTAGGT CGGCCAGATG GGTGGATGGA CTACCAGAAA CGGTATGGAG GTTCTCTGAA	720
35	GCGCTTTGOC GAGGCTCTGC CCTCGGAGA AGAAGGOGAA AGTTACTCCA AAGAAGTTC	780
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(2) INFORMATION FOR SEQ ID NO:30:

40 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 598 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 45 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- 90 -

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

5

(vii) IMMEDIATE SOURCE:

(B) CLONE: IRES sequence

10

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 1..598

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

	GAATTCGGCC CCTCTCCCTC CCCCCCCCCT AACGTTACTG GCGAAGCGG CTTCGAATAA	60
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20	AGGGCCCGGA AACCTGGCC TGCTCTCTG ACGAGCATC CTAGGGGCTT TCCCCCTC	180
	GCGAAGGAA TGCAAGGCT GTTGATGTC GTGAGGAAG CAGTCTCTT GGAAGCTTCT	240
25	TGAGACAAA CAAGTCTGT AGGACCTTT TGAGGCAGC GGAACCCCC AACCTGGGAC	300
	AGGTCCTCT GGGGCAAAA GCGAGGTGA TAGATACAC CTGCAAGGC GGCACAAAC	360
	CAGTCCACG TTGTGAGTG GATAGTGTG GAAAGATCA AATGGCTCT CTCACGGTA	420
30	TTCAACAAGG GCGTGAAGA TGGCAGAAG GTACCCATT GTATGGGATC TGATCTGGG	480
	CTCTGGTGA CATGCTTAC ATGTGTTAG TCGAGGTAA AAAAGCTCA GGGCCCCGA	540
35	AACAGGGGA CGGGTTTTC CTTGAAAAA CAGATGATA AGCTTGGAC AACCATGG	598

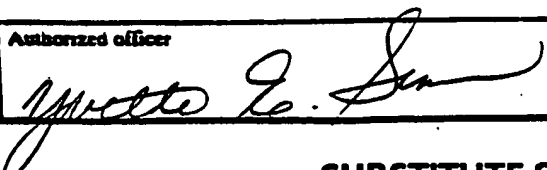
90/1

Applicant's or agent's file reference number	CTI/29 CIP PCT	International application No.
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>54</u> line <u>S 14-23</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution American Type Culture Collection	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 United States of America	
Identification Reference by Depositor: Cell Line, RINa/ProA/ P030/P088	
Date of deposit 07 June 1995 (07.06.95)	Accession Number CRL 11921
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input checked="" type="checkbox"/>	
In respect of the designation of the EPO, samples of the deposited microorganisms will be made available until the publication of the mention of the grant of the European patent or until the date on which the application is refused or withdrawn or is deemed to be withdrawn, as provided in Rule 28(3) of the Implementing Regulations under the EPC only by the issue of a sample to an expert nominated by requester (Rule 28(4) EPC)	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
EPO	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

For receiving Office use only	For International Bureau use only
<input checked="" type="checkbox"/> This sheet was received with the international application	<input type="checkbox"/> This sheet was received by the International Bureau on:
Authorized officer 	Authorized officer

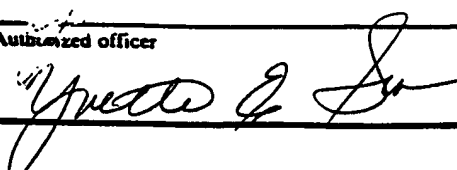
SUBSTITUTE SHEET (RULE 26)

90/2

Applicant's or agent's file reference number	CTI/29 CIP PCT	International application No.
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>54</u> , line S <u>14-23</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution American Type Culture Collection	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 United States of America	
Cell Line, RINa/ProA/ Identification Reference by Depositor: P030/P088	
Date of deposit 07 June 1995 (07.06.95)	Accession Number CRL 11921
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input checked="" type="checkbox"/>	
In respect of the designation of Finland, until the application has been laid open to public inspection by the Finnish Patent Office, or has been finally decided upon by the Finnish Patent Office without having been laid open to public inspection, samples of the deposited microorganisms will be made available only to an expert in the art.	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
Finland	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
For receiving Office use only	For International Bureau use only
<input checked="" type="checkbox"/> This sheet was received with the international application	<input type="checkbox"/> This sheet was received by the International Bureau on:
Authorized officer 	Authorized officer

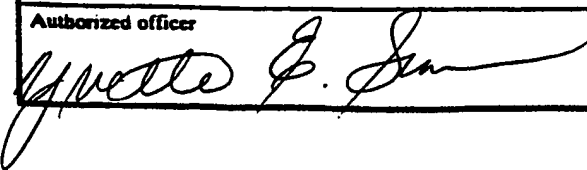
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Applicant's or agent's file reference number	CTI/29 CIP PCT	International application No.
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made herein relate to the microorganism referred to in the description on page <u>54</u> line <u>S 14-23</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution American Type Culture Collection	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 United States of America	
Cell Line, RiNa/ProA/ Identification Reference by Depositor: P030/P088	
Date of deposit 07 June 1995 (07.06.95)	Accession Number CRL 11921
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
Applicant(s) hereby give notice of my/our intention that samples of the above-identified culture shall be available only to experts in accordance with paragraph 3 of the Fourth Schedule to the Patents Rules 1995.	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
Singapore	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

For receiving Office use only
<input checked="" type="checkbox"/> This sheet was received with the international application
Authorized officer 

For International Bureau use only
<input type="checkbox"/> This sheet was received by the International Bureau on:
Authorized officer

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WE CLAIM:

1. A cell stably transformed to produce at least one analgesic compound from each of the groups consisting of endorphins, enkephalins, and catecholamines.

2. The cell of claim 1, wherein the endorphin is β -endorphin.

3. The cell of claim 1, wherein the enkephalin is met-enkephalin.

4. The cell of claim 1, wherein the catecholamine is norepinephrine or epinephrine.

5. The cell of any one of claims 1-4 wherein the cell is a RIN cell.

6. The cell of any one of claims 1-4 wherein the cell is an AtT-20 cell.

7. The cell of any one of claims 1-6 wherein the cell additionally produces a compound selected from the group consisting of galanin, somatostatin, neuropeptide Y, neurotensin, or cholecystokinin.

8. A cell transformed with a DNA encoding POMC, a DNA encoding TH, a DNA encoding DBH, and a DNA encoding ProA, each DNA molecule operably linked to an expression control sequence.

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9. The cell of claim 8 wherein the cell is transformed with pCEP4-POMC-030, pcDNA3-hproA+KS-091, and pZeo-pCMV-rTHAKS-IRES-bDBH-088.

10. The cell of claim 8 wherein the cell is transformed with pCEP4-h POMC- Δ ACTH-032, pBS-CMV-proA, and pZeo-pCMV-rTHAKS-IRES-bDBH-088.

11. The cell of claim 8 wherein the cell is transformed with pcDNA3-hPOMC Δ ACTH-IRES-rTHD-IRES-bDBH-IRES-Zeocin-073 and pcDNA3-proA+KS-091.

12. A transformed cell producing at least one enkephalin, one endorphin and one catecholamine, wherein the cell is transformed with:

a first vector containing a DNA encoding POMC operably linked to an expression control sequence,

a second vector containing a DNA encoding pro-enkephalin A operably linked to an expression control sequence,

a third vector containing a DNA encoding TH operably linked to an expression control sequence and a DNA encoding dopamine beta hydroxylase operably linked to an expression control sequence.

13. A method for treating pain comprising implanting at an implantation site in a patient a therapeutically effective number of the cells of any of claims 1-12.

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14. The method of claim 13 wherein the cells are encapsulated in a semi-permeable membrane to form a bioartificial organ.

15. The method of claim 14 wherein the bioartificial organ is immunoisulatory.

16. The method of any one of claims 13-15 wherein the implantation site is the CNS.

17. The method of any one of claims 13-15 wherein the implantation site is the sub-arachnoid space.

18. A method of producing a cell that secretes at least one enkephalin, one endorphin and one catecholamine, comprising transforming the cell with a DNA encoding POMC operably linked to a first expression control sequence, a DNA encoding pro-enkephalin A operably linked to a second expression control sequence, and a DNA encoding TH operably linked to a third expression control sequence and a DNA encoding dopamine beta hydroxylase operably linked to a fourth expression control sequence.

19. The method of claim 18 wherein said first, second, third and fourth expression control sequences are identical.

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20. The use of the cells of any of claims 1-12 to manufacture a medicant for treatment of pain.

21. The cells of claim 20 wherein the cells are implanted.

22. The cells of any one of claims 21-22 wherein the cells are encapsulated in a semi-permeable membrane to form a bioartificial organ.

23. The cells of claim 22 wherein the bioartificial organ is immunoisulatory.

24. The cells of any one of claims 21-23 wherein the implantation site is the CNS.

25. The cells of any one of claims 21-23 wherein the implantation site is the sub-arachnoid space.

26. A bioartificial organ comprising:

(a) a biocompatible, permeable jacket surrounding a core; and

(b) said core comprising at least one living cell transformed to produce at least one analgesic compound from each of the groups consisting of endorphins, enkephalins, and catecholamines.

27. The bioartificial organ of claim 26 for use in treating pain.

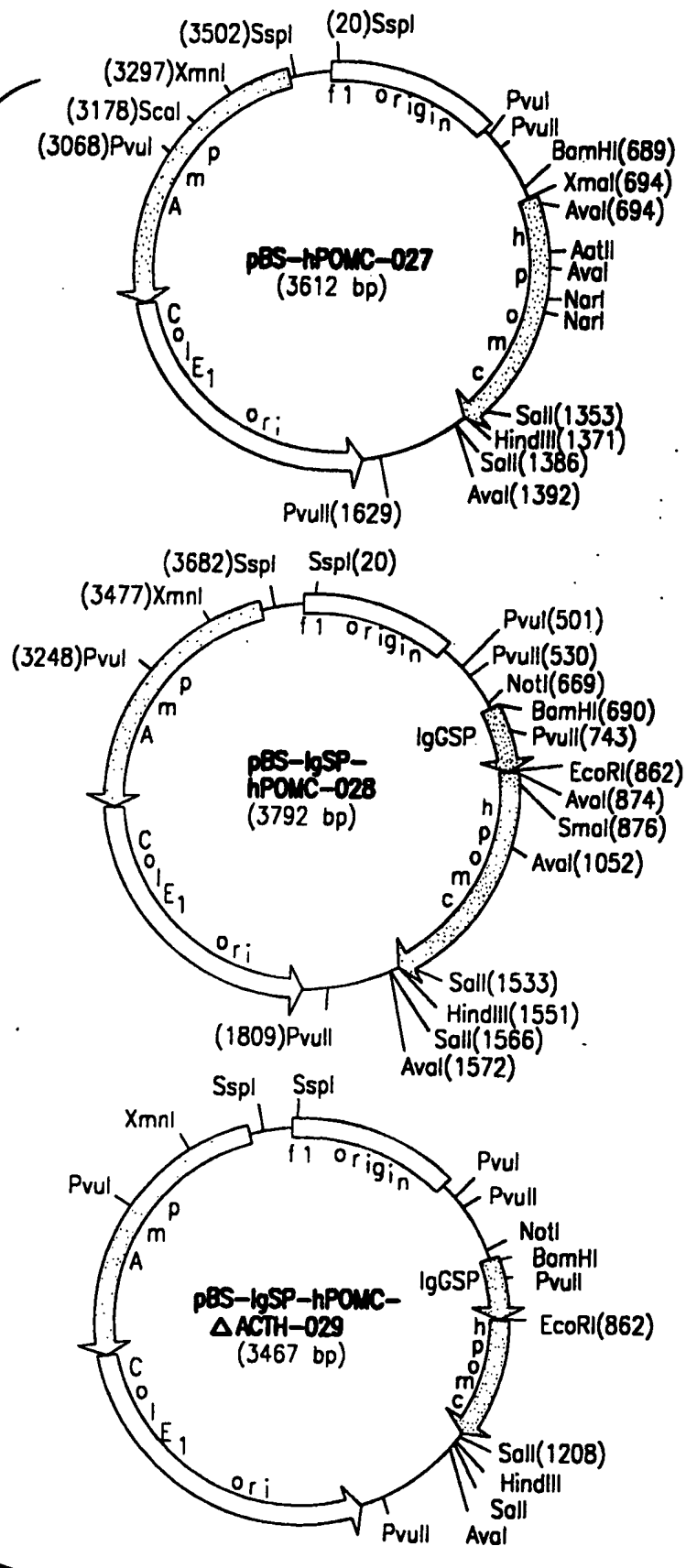
- 95 -

28. A method of making a bioartificial organ comprising encapsulating a core comprising at least one living cell transformed to produce at least one analgesic compound from each of the groups consisting of endorphins, enkephalins, and catecholamines, with a biocompatible, permeable jacket.

29. The use of a bioartificial organ comprising the cells of claims 1-12 in manufacture of a medicament for treating of pain.

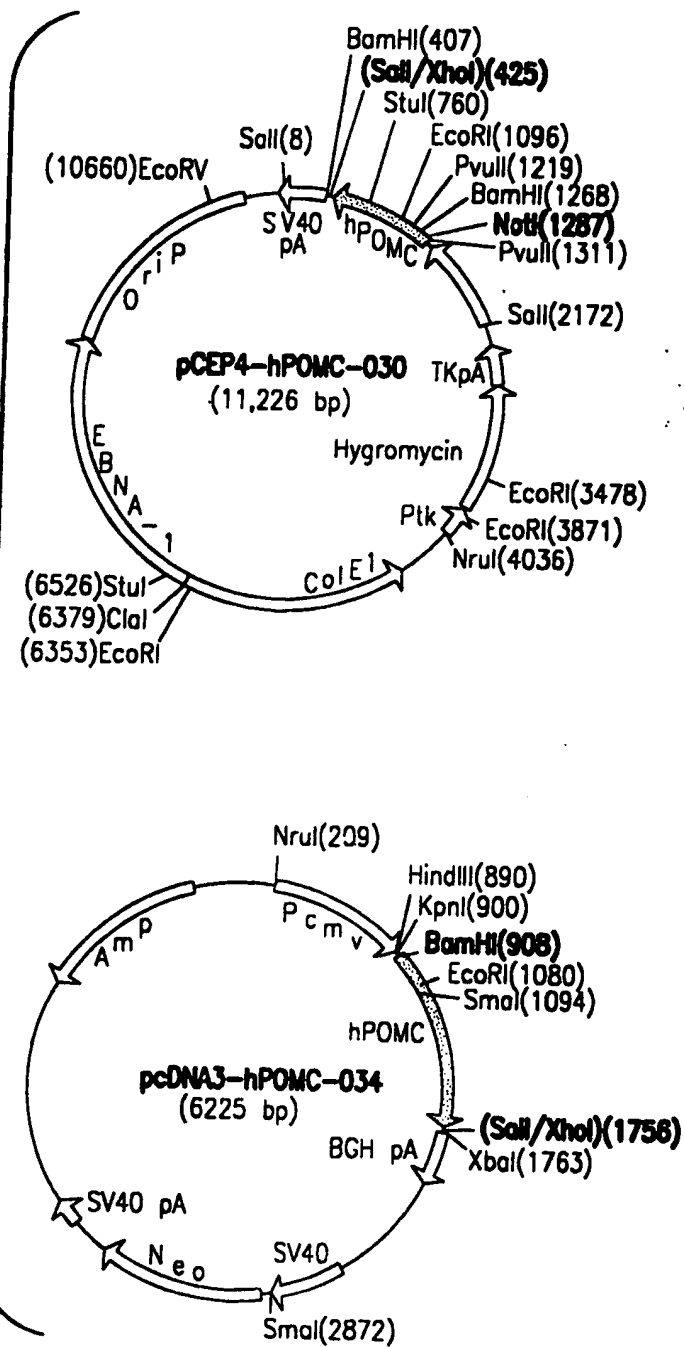
1 / 13

FIG. 1



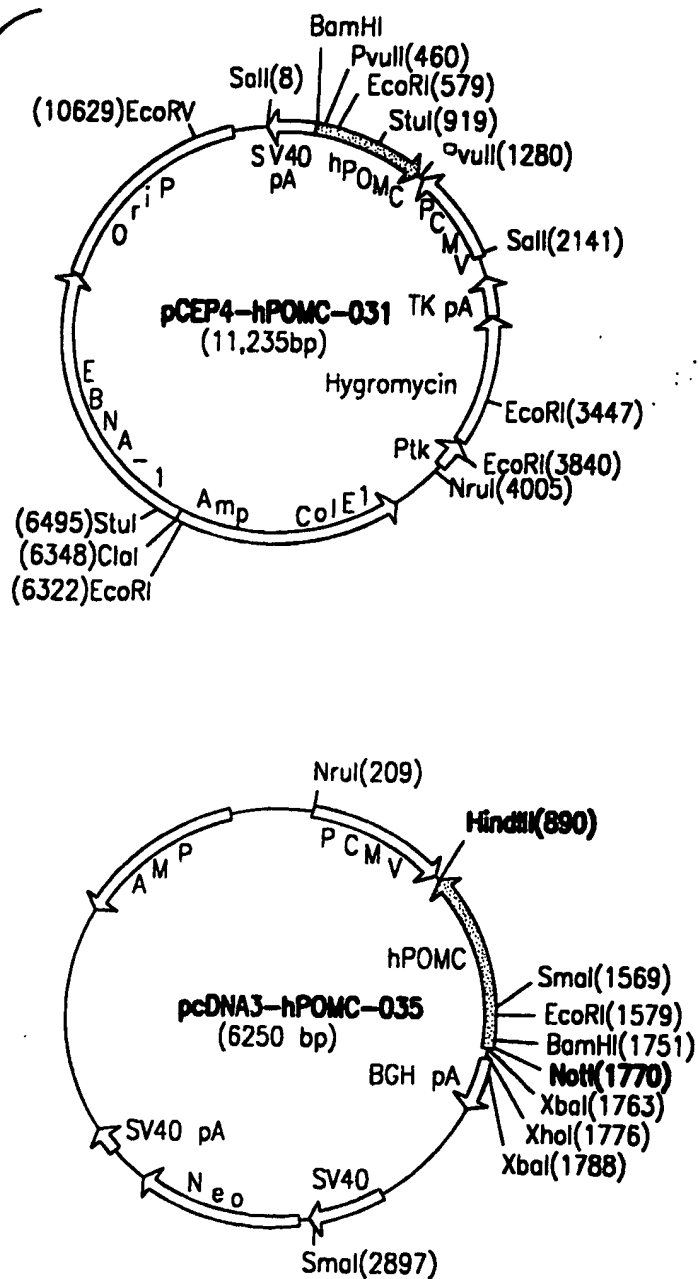
2 / 13

FIG. 2a



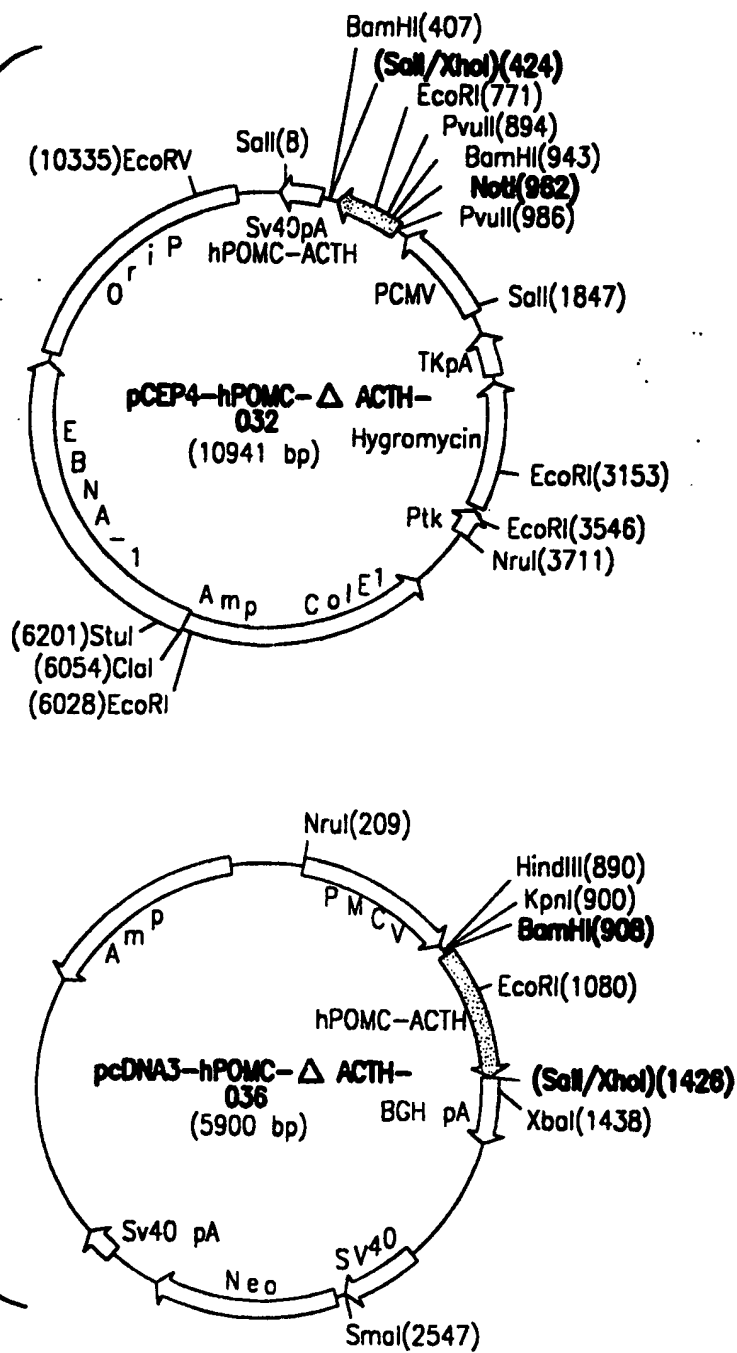
3 / 13

FIG. 2b



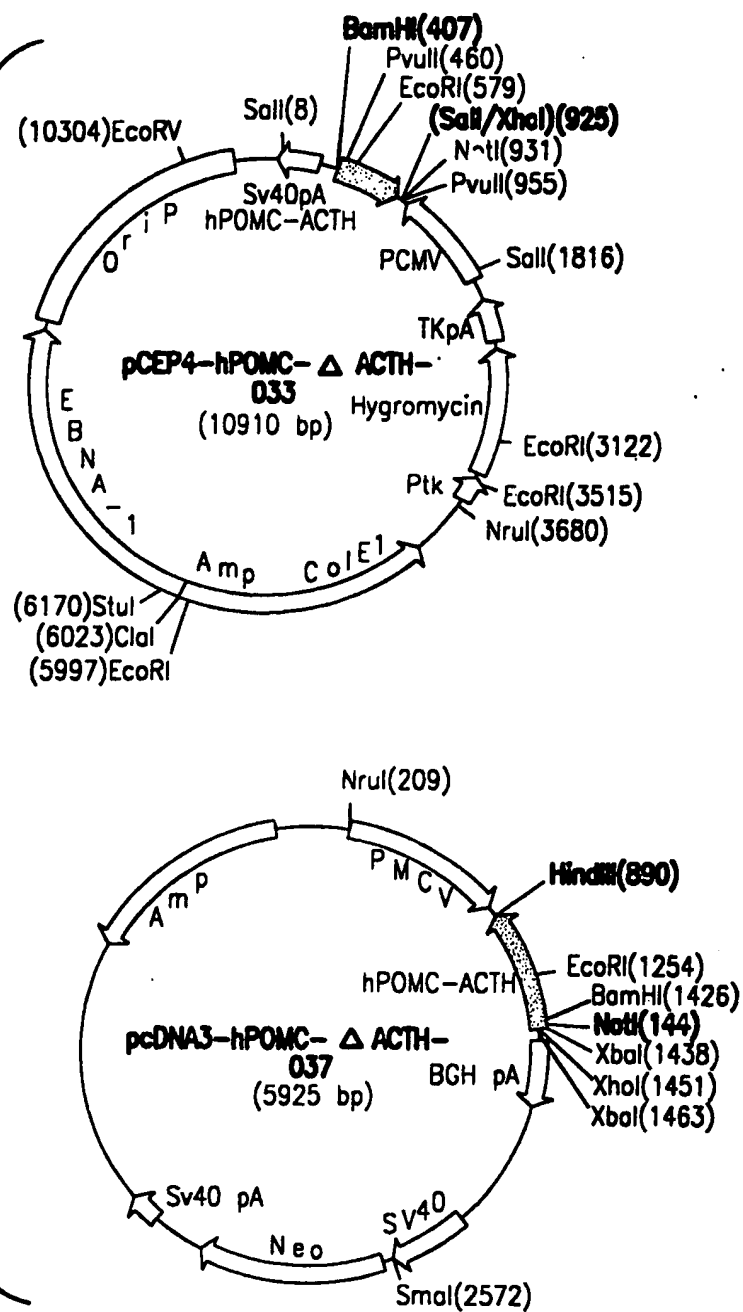
4 / 13

FIG. 3a



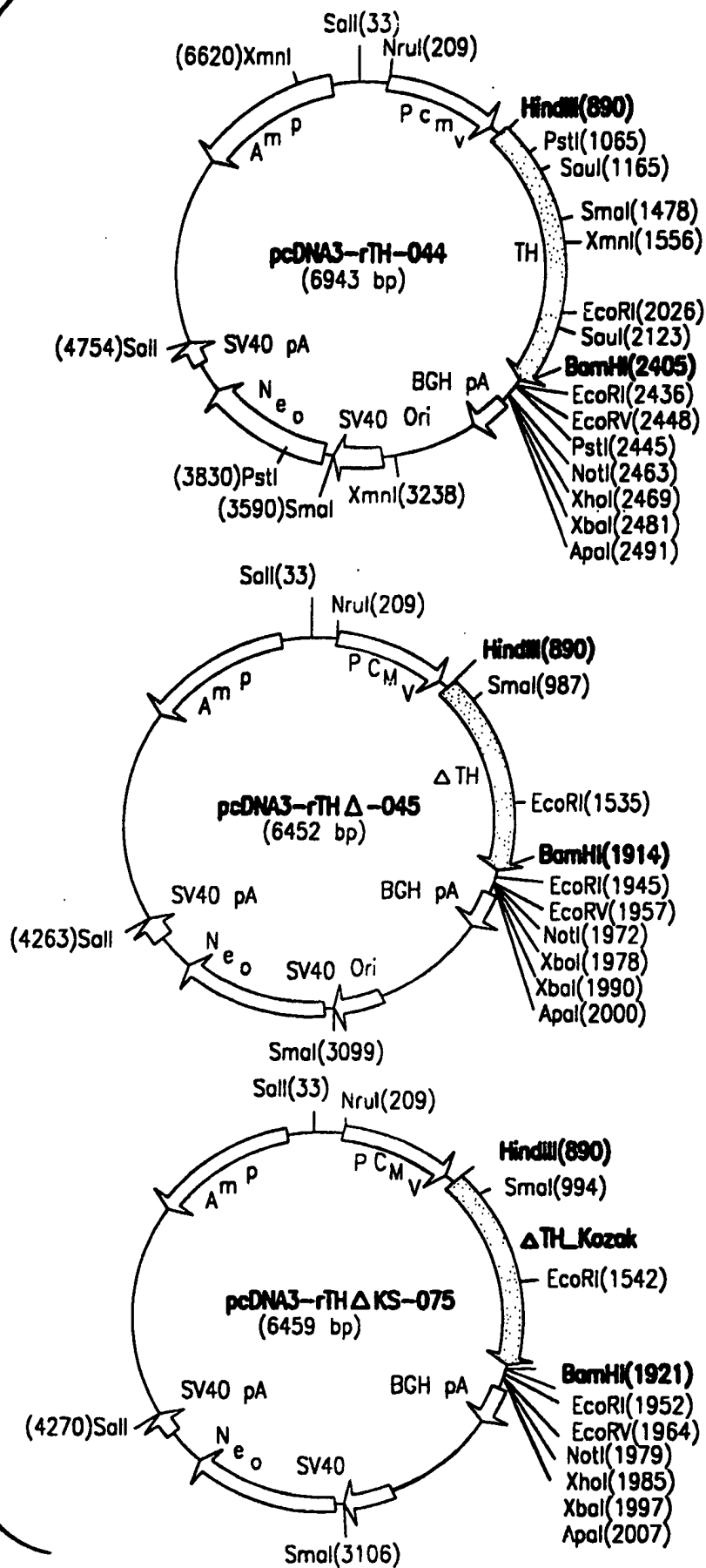
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FIG. 3b



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FIG. 4



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FIG. 5

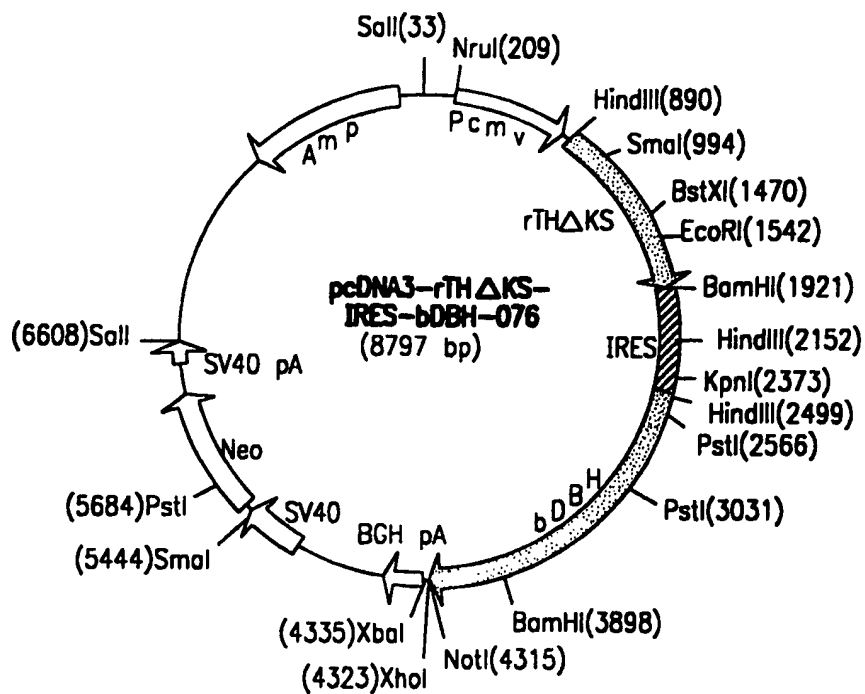
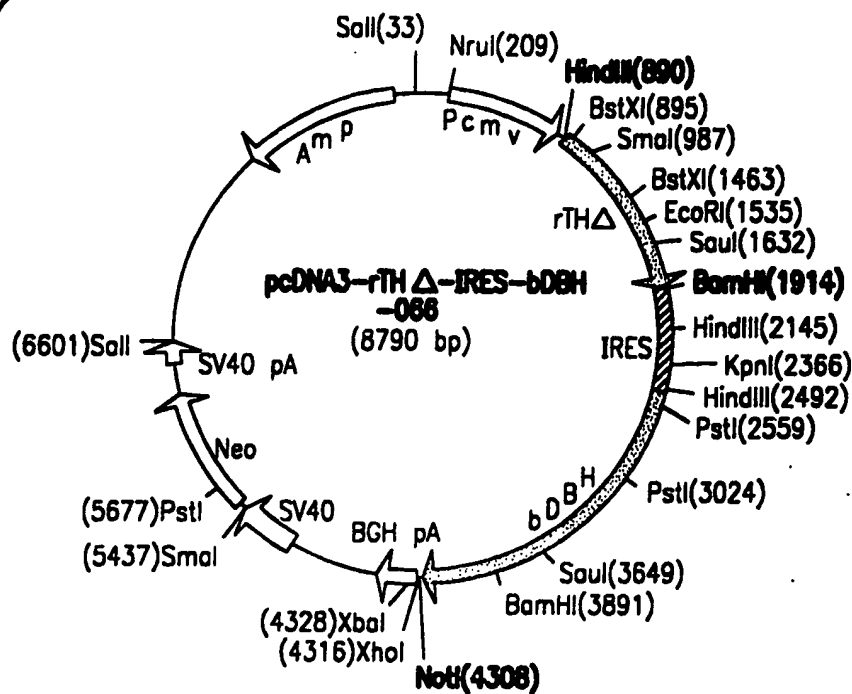
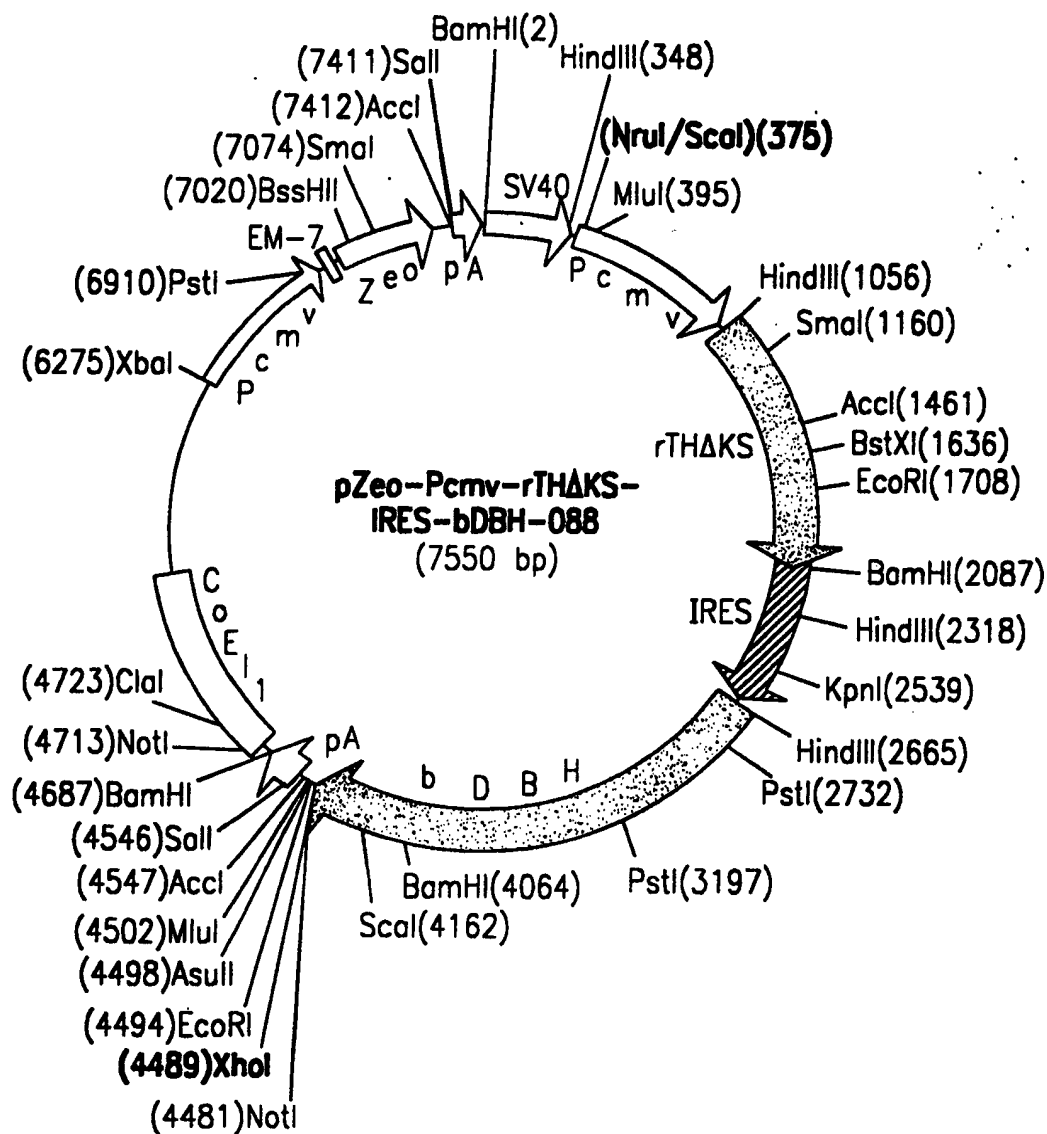


FIG. 6



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FIG. 7

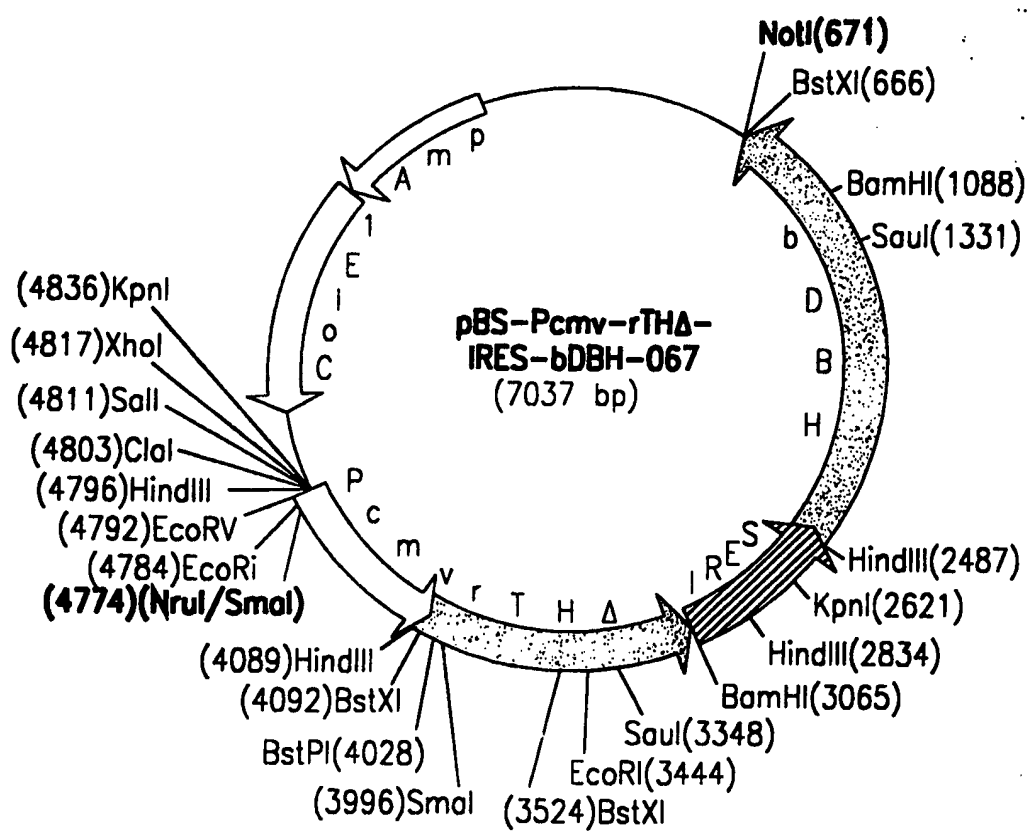
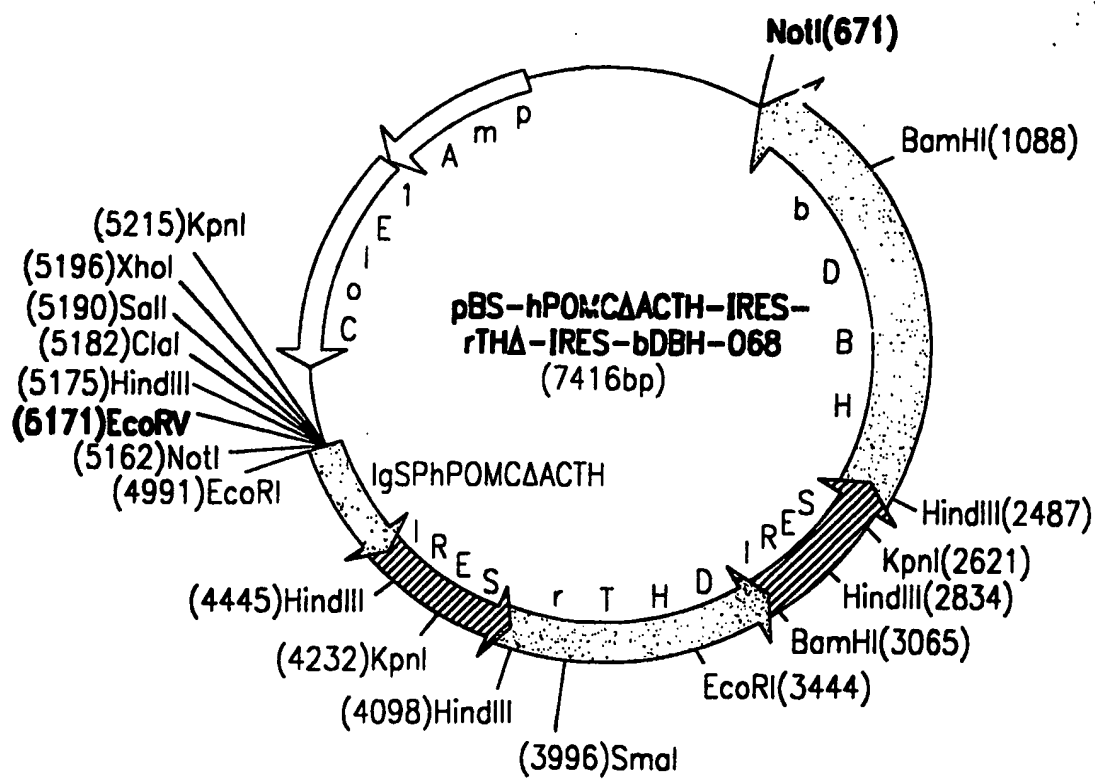
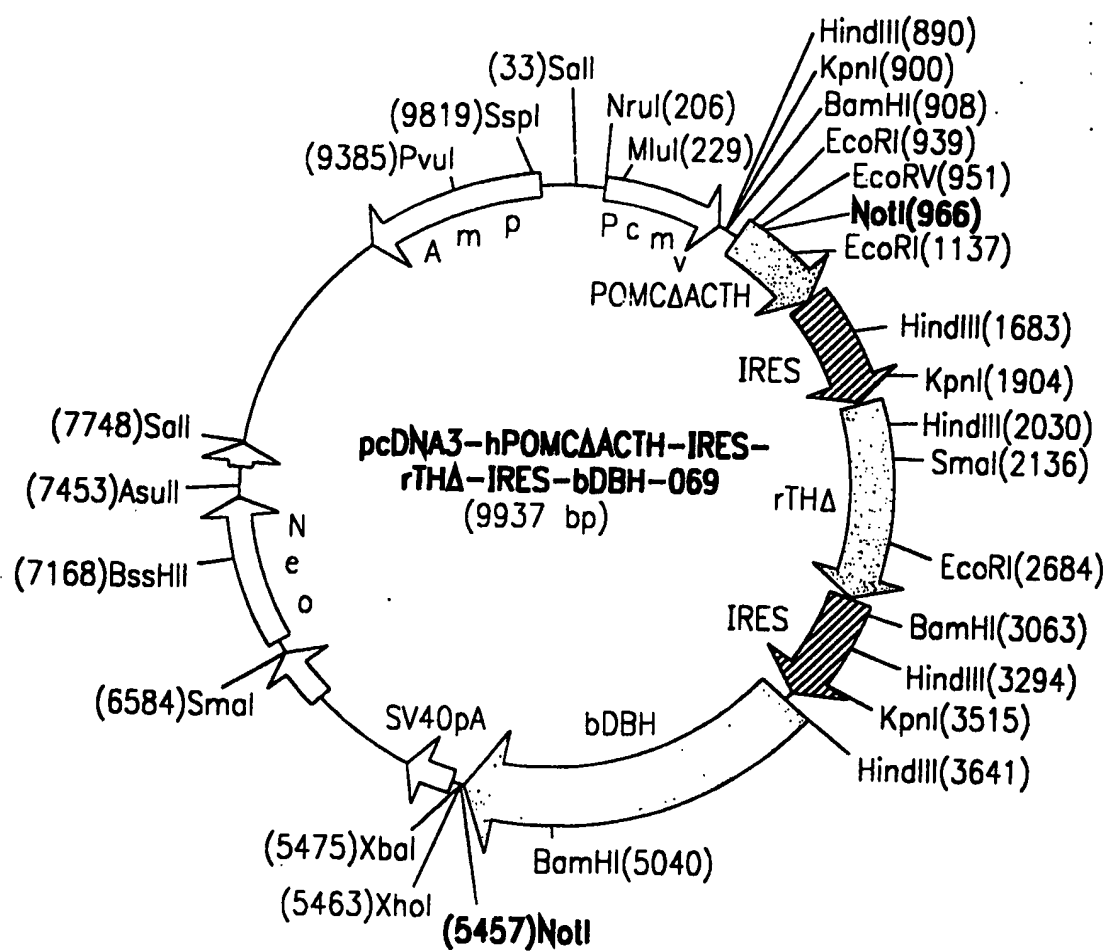


FIG. 8



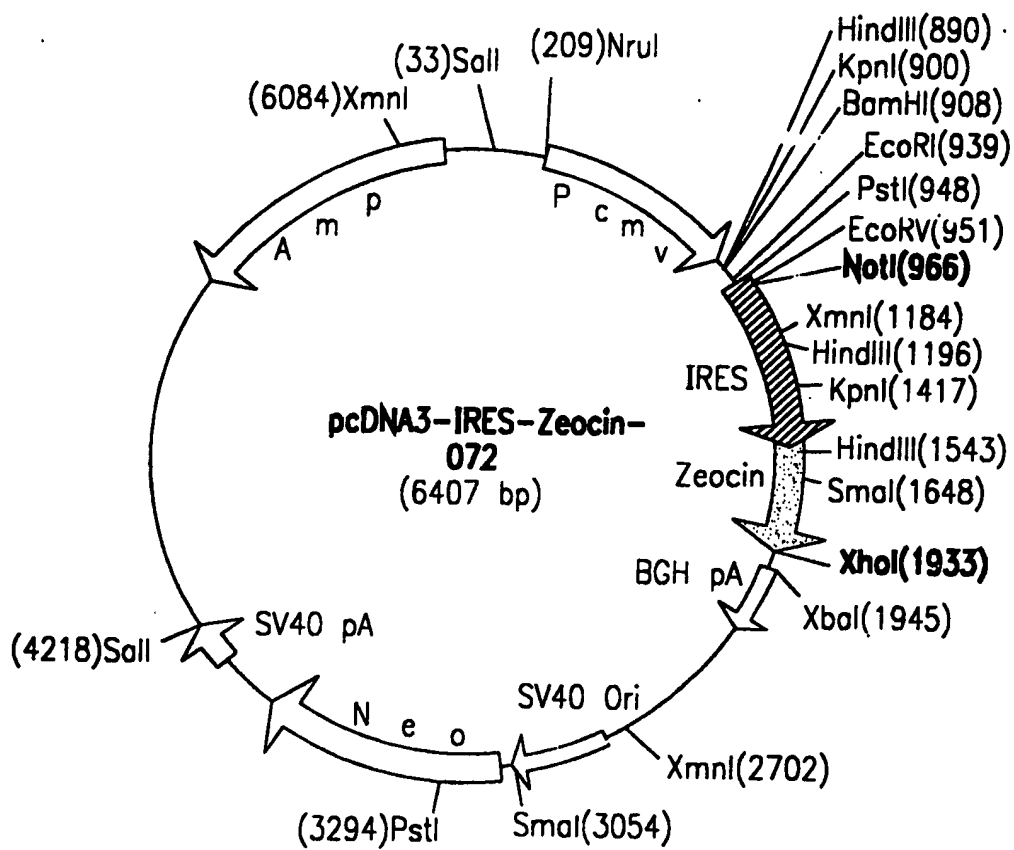
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FIG. 9



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FIG. 10



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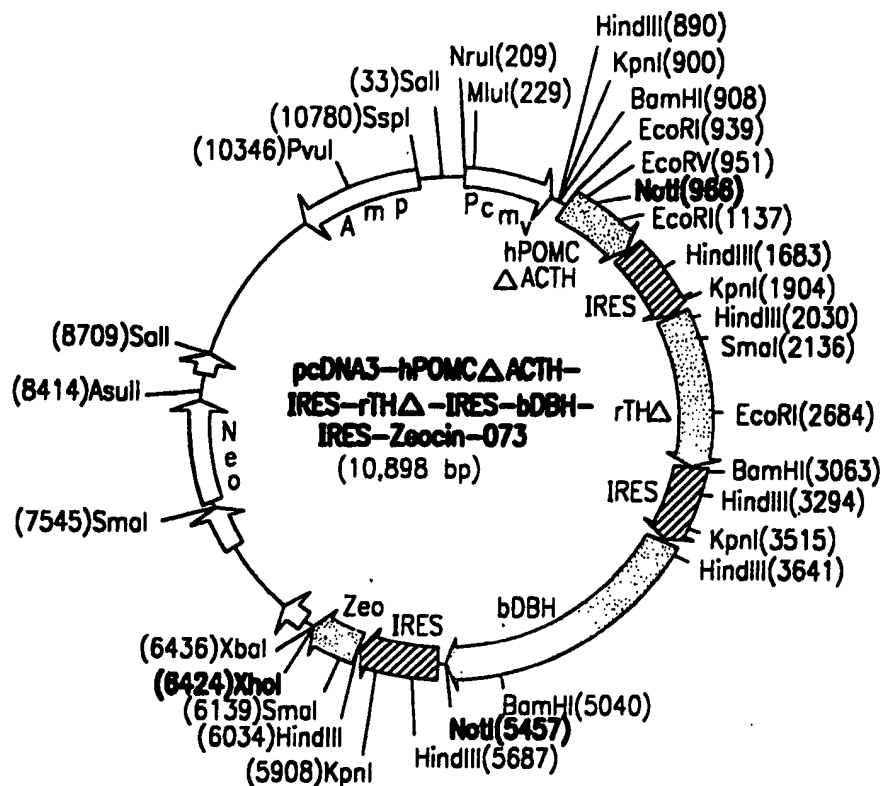


FIG. 11

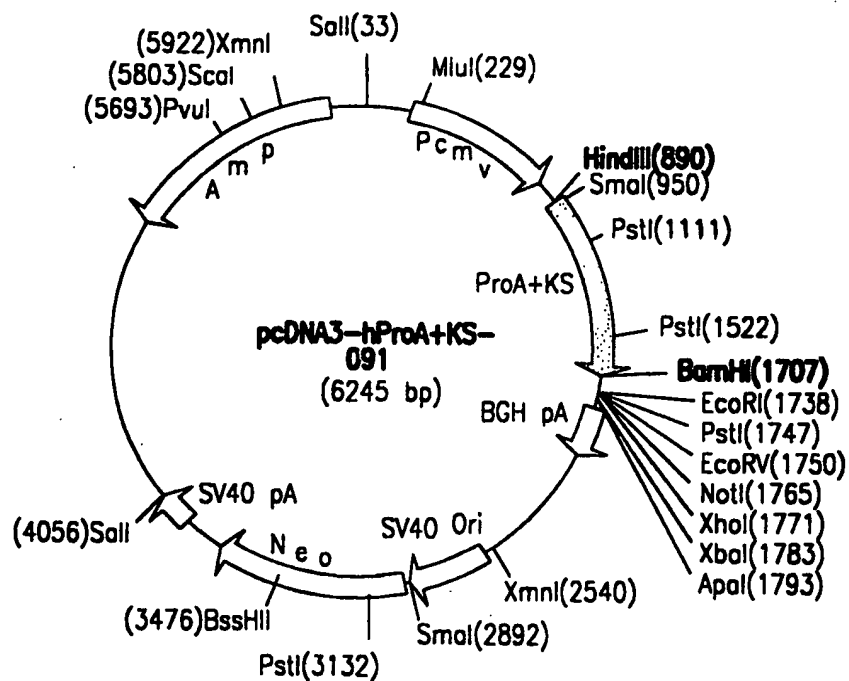


FIG. 12

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/09629

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N15/87 C12N5/10 A61K9/48 A61K38/16 A61K38/33		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,95 05452 (CYTOTHERAPEUTICS, INC.) 23 February 1995 see the whole document, especially pages 12-31 and Example 6.	1-4,8, 12-29
A	J. NEUROSCI., vol. 14, 1994, pages 4806-4814, XP002018157 H.H. WU ET AL.: "Implantation of AtT-20 or genetically modified AtT-20/hENK cells in mouse spinal cord induced antinociception and opioid tolerance" cited in the application see the discussion.	1
<div style="display: flex; justify-content: space-around;"> <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex. </div>		
<div style="display: flex;"> <div style="flex: 1;"> <p>* Special categories of cited documents :</p> <p>*A* document defining the general state of the art which is not considered to be of particular relevance</p> <p>*E* earlier document but published on or after the international filing date</p> <p>*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>*O* document referring to an oral disclosure, use, exhibition or other means</p> <p>*P* document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="flex: 1;"> <p>*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>*&* document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search <div style="text-align: center; font-size: 1.2em;">14 November 1996</div>		Date of mailing of the international search report <div style="text-align: center; font-size: 1.2em;">28.11.96</div>
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016		Authorized officer <div style="text-align: center; font-size: 1.2em;">Yeats, S</div>

INTERNATIONAL SEARCH REPORT

In International Application No
PCT/US 96/09629

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>PROC. NATL. ACAD. SCI. USA, vol. 83, 1986, pages 7522-7526, XP002018158 J. SAGEN ET AL.: "Analgesia induced by isolated bovine chromaffin cells implanted in rat spinal cord" cited in the application see the abstract and discussion.</p> <p style="text-align: center;">---</p>	1
A	<p>NATURE, vol. 297, 1982, pages 335-339, XP002018159 M. COCHET ET AL.: "Characterization of the structural gene and putative 5'-regulatory sequences for human proopiomelanocortin" cited in the application see the whole document.</p> <p style="text-align: center;">-----</p>	1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/09629

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 13-17
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 13-17 are directed to a method for treatment of the human body by therapy (Rule 39 PCT), the search has been carried out based on the alleged effects of the composition mentioned in the claims.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/09629

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9505452	23-02-95	AU-A- 7568094	14-03-95
		CA-A- 2169292	23-02-95
		FI-A- 960611	09-04-96
		NO-A- 960547	12-04-96
